LIST OF REFERENCES CITED BY APPLICANT (Use several sheets if necessary)		
	Application Number	12/466,213
	Filing Date	May 14, 2009
	First Named Inventor	Jeffrey B. ETTER
	Art Unit	1623
	Examiner Name	CRANE, Lawrence E.
	Attorney Docket No.	9516-847-999
	CAM No.	501872-999847

U.S. PATENT and PUBLICATION DOCUMENTS

*Examiner Initials		Document Number	Date yyyy-mm-dd	Name of Patentee or Applicant of Cited Document	
	A48	US 2012/0196823	2012-08-02	Tutino et al.	
	A49	US 2013/0109644	2013-05-02	MacBeth et al.	

FOREIGN PATENT DOCUMENTS

*Examiner Initials		Foreign Patent Document Country Code, Number, Kind Code (if known)	Date yyyy-mm-dd	Name of Patentee or Applicant of Cited Document		T
	B13	WO 2004/082822	2004-09-30	Pharmion Corporation		
	B14	WO 2006/089290	2006-08-24	American Bioscience Inc.		
	B15	WO 2009/139888	2009-11-19	Celgene Corporation		
	B16	WO 2010/059969	2010-05-27	Genentech Inc.		
	B17	WO 2012/135405	2012-10-04	Pharmion LLC		
	B18	WO 2013/022872	2013-02-14	Celgene Corporation		

NON PATENT LITERATURE DOCUMENTS

*Examiner Initials		Include name of the author (in CAPITAL LETTERS), (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T
	C72	ANONYMOUS, "A Phase I/II Clinical Trial of Vidaza with Abraxane in the Treatment of Patients with Advanced or Metastatic Solid Tumors and Breast Cancer," ClinicalTrials.gov archive, pages 1-3, retrieved from the Internet: http://clinicaltrials.gov/archive/NCT00748553/2011_08_05, on January 31, 2013	
	C73	ANONYMOUS, "Oral Azacitidine as a Single Agent and in Combination with Carboplatin or Abraxane in Subjects with Relapsed or Refractory Solid Tumors," ClinicalTrials.gov archive, pages 1-4, retrieved from the Internet: http://clinicaltrials.gov/archive/NCT01478685/2011_11_22, on January 31, 2013	
	C74	BAST et al., "A Phase IIa Study of a Sequential Regimen Using Azacitidine to Reverse Platinum Resistance to Carboplatin in Patients with Platinum Resistant or Refractory Epithelial Ovarian Cancer," Journal of Clinical Oncology, 26, Abstract 3500, (2008)	
	C75	COWAN et al., "Will DNA Methylation Inhibitors Work in Solid Tumors? A Review of the Clinical Experience with Azacitidine and Decitabine in Solid Tumors," Epigenomics, Future Medicine Ltd, United Kingdom, 2(1):71-86 (2010)	
	C76	GLASER, "HDAC Inhibitors: Clinical Update and Mechanism-Based Potential," Biochem. Pharm., 74:659–71 (2007)	

SDI-162357v1

EXAMINER	DATE CONSIDERED

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

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	C77	HOWELL et al., "Demethylating Agents in the Treatment of Cancer," Pharmaceuticals, 3(7):2022–44 (2010)	
	C78	JUERGENS et al., "Interim Analysis of a Phase II Trial of 5-Azacitidine (5AC) and Entinostat (SNDX-275) in Relapsed Advanced Lung Cancer (NSCLC)," Journal of Clinical Oncology, 27(15S):8055 (2009)	
	C79	MOMPARLER, "Epigenetic Therapy of Cancer with 5-Aza-2'-Deoxycitidine (Decitabine)," Seminars in Oncology, 32(5):443–51 (2005)	
	C80	STATHIS et al., "Phase I Study of Decitabine in Combination with Vorinostat in Patients with Advanced Solid Tumors and Non-Hodgkin's Lymphomas," Clinical Cancer Research, 17(6):1582–90 (2011)	
	C81	Vidaza™ Label (azacitidine for injectable suspension), Version: 5-18-04.	
	C82	Vidaza™ (azacitidine for injection) Prescribing Information, dated January 2012.	

SDI-162357v1

EXAMINER	DATE CONSIDERED
*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.	

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
30 September 2004 (30.09.2004)

PCT

(10) International Publication Number
WO 2004/082822 A2

(51) International Patent Classification[7]: B01J

(21) International Application Number:
PCT/US2004/007896

(22) International Filing Date: 16 March 2004 (16.03.2004)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
10/390,530 17 March 2003 (17.03.2003) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

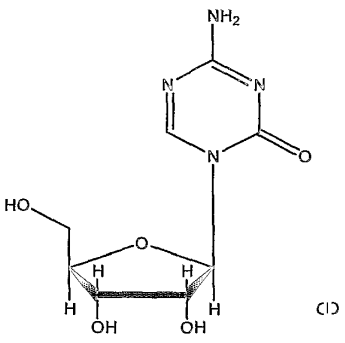
(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2004/082822 A2

(54) Title: METHODS FOR ISOLATING CRYSTALLINE FORM I OF 5-AZACYTIDINE



(I)

(57) Abstract: The invention includes methods for isolating crystalline Form I of 5-azacytidine substantially free of other forms, wherein 5-azacytidine is represented by the formula: The invention also includes pharmaceutical compositions comprising Form I of 5-azacytidine.

METHODS FOR ISOLATING CRYSTALLINE FORM I OF 5-AZACYTIDINE

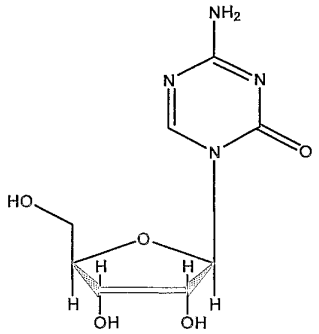
Field of the Invention

The invention relates to the isolation of crystalline polymorphic Form I of 5-azacytidine (also known as azacitidine and 4-amino-1-β-D-ribofuranosyl-S-triazin-2(1*H*)-one). 5-azacytidine may be used in the treatment of disease, including the treatment of myelodysplastic syndromes (MDS).

Background of the Invention

Polymorphs exist as two or more crystalline phases that have different arrangements and/or different conformations of the molecule in a crystal lattice. When a solvent molecule(s) is contained within the crystal lattice the resulting crystal is called a pseudopolymorph, or solvate. If the solvent molecule(s) within the crystal structure is a water molecule, then the pseudopolymorph/solvate is called a hydrate. The polymorphic and pseudopolymorphic solids display different physical properties, including those due to packing, and various thermodynamic, spectroscopic, interfacial and mechanical properties (See H. Brittain, Polymorphism in Pharmaceutical Solids, Marcel Dekker, New York, NY, 1999, pp. 1-2). Polymorphic and pseudopolymorphic forms of the drug substance (also known as the "active pharmaceutical ingredient" (API)), as administered by itself or formulated as a drug product (also known as the final or finished dosage form, or as the pharmaceutical composition) are well known and may affect, for example, the solubility, stability, flowability, fractability, and compressibility of drug substances and the safety and efficacy of drug products, (see, *e.g.*, Knapman, K Modern Drug Discoveries, March 2000: 53).

5-Azacytidine (also known as azacitidine and 4-amino-1-β-D-ribofuranosyl-S-triazin-2(1*H*)-one; Nation Service Center designation NSC-102816; CAS Registry Number 320-67-2) has undergone NCI-sponsored trials for the treatment of myelodysplastic syndromes (MDS). *See* Kornblith *et al.*, J. Clin. Oncol. 20(10): 2441-2452 (2002) and Silverman *et al.*, J. Clin. Oncol. 20(10): 2429-2440 (2002). 5-azacytidine may be defined as having a formula of $C_8H_{12}N_4O_5$, a molecular weight of 244.20 and a structure of:



In the United States Patent Application Serial No. 10/390,578 entitled "Forms of 5-azacytidine," filed March 17, 2003 and incorporated herein by reference in its entirety, eight different polymorphic and pseudopolymorphic forms of 5-azacytidine (Forms I-VIII), in addition to an amorphous form, are described. Forms I-VIII each have characteristic X-Ray Powder Diffraction (XRPD) patterns and are easily distinguished from one another using XRPD.

5-azacytidine drug substance used in the previous clinical trials has typically been synthesized from 5-azacytosine and 1,2,3,5,-tetra-O-acetyl-β-D-ribofuranose by the method presented in Example 1. The last step of this method is a recrystallization of the crude synthesis product from a methanol/DMSO co-solvent system. Specifically, the crude synthesis product is dissolved in DMSO (preheated to about 90°C), and then methanol is added to the DMSO solution. The product is collected by vacuum filtration and allowed to air dry.

In In the United States Patent Application Serial No. 10/390,578 entitled "Forms of 5-azacytidine," filed March 17, 2003 and incorporated herein by reference in its entirety, it is demonstrated that this prior art method for the recrystallization of the crude synthesis product does not control for the polymorphic forms of 5-azacytidine. Specifically, the prior art recrystallization procedure produces either Form I substantially free of other forms, or a Form I/II mixed phase *i.e.* a solid material in which 5-azacytidine is present in a mixed phase of both polymorphic Form I and polymorphic Form II. Thus, the prior art procedures do not allow one to reliably target Form I as the single polymorphic form in the drug substance. The present invention provides methods that allow one to recrystallize 5-azacytidine as polymorphic Form I robustly and reproducibly.

Summary of the Invention

The present invention provides methods for robustly and reproducibly isolating 5-azacytidine as polymorphic Form I substantially free of other forms. The methods involve recrystallizing dissolved 5-azacytidine from a primary solvent/co-solvent mixture and then collecting the resultant crystals. The invention also provides pharmaceutical compositions comprising Form I of 5-azacytidine together with a pharmaceutically acceptable excipient, diluent, or carrier.

Detailed Description of the Preferred Embodiments

Polymorphic Form I of 5-azacytidine

Form I of 5-azacytidine is described in United States Patent Application Serial No. 10/390,578 entitled "Forms of 5-azacytidine," filed March 17, 2003 and incorporated herein by reference in its entirety. Table 1 provides the most prominent 2θ angles, d-spacing and relative intensities for Form I observed using X-Ray Powder Diffraction (XRPD) performed according the method of Example 4:

2θ Angle (°)	*d-spacing (Å)*	*Relative Intensity*
12.182	7.260	39.1
13.024	6.792	44.1
14.399	6.146	31.5
16.470	5.378	27.1
18.627	4.760	16.0
19.049	4.655	35.9
20.182	4.396	37.0
21.329	4.162	12.4
23.033	3.858	100.0
23.872	3.724	28.0
26.863	3.316	10.8
27.135	3.284	51.5
29.277	3.048	25.6
29.591	3.016	11.5
30.369	2.941	10.8
32.072	2.788	13.4

Table 1: 5-azacytidine Form I - the most prominent 2θ angles, d-spacing and relative intensities (Cu Kα radiation)

Isolation of Polymorphic Form I of 5-azacytidine by Recrystallization

Form I of 5-azacytidine may be reproducibly isolated substantially free of other forms by recrystallizing dissolved 5-azacytidine and collecting the resultant crystals. Specifically, 5-azacytidine is first dissolved completely in at least one suitable primary solvent, preferably a polar solvent, more preferably a polar aprotic solvent. Suitable polar aprotic solvents include, but are not limited to, dimethylformamide (DMF), dimethylacetamide (DMA), dimethylsulfoxide (DMSO), and N-methylpyrrolidinone (NMP). The most preferred polar aprotic solvent is DMSO. Mixtures of two or more primary solvents are also contemplated for dissolving the 5-azacytidine, for example a mixture of DMSO and DMF.

The 5-azacytidine used to form the solution may be synthesized by any procedure known in the art; an exemplary prior art synthesis scheme is provided in Example 1. Any polymorphic or pseudopolymorphic form(s) of 5-azacytidine, including mixed phases, may be used to form the solution. Amorphous 5-azacytidine may also be used to form the solution. It is preferred, but not required, that the primary solvent is preheated to an elevated temperature in order to ensure that the 5-azacytidine is dissolved completely. An especially preferred primary solvent is dimethyl sulfoxide, (DMSO), most preferably preheated to a temperature in the range of about 40°C to about 90°C.

Following solvation of the 5-azacytidine in the primary solvent, at least one co-solvent is added to the solution of 5-azacytidine. Suitable co-solvents include C_2-C_5 alcohols (which term hereinafter refers to C_2-C_5 alcohols that are independently: branched or unbranched, substituted or unsubstituted), aliphatic ketones (which term hereinafter refers to aliphatic ketones that are indepedently: branched or unbranched, substituted or unsubstituted), and alkyl cyanides (which term hereinafter refers to alkyl cyanides that are independently: branched or unbranched, substituted or unsubstituted). Preferred C_2-C_5 alcohols, aliphatic ketones, and alkyl cyanides, along with other suitable solvents, are listed below as Class 2 (solvents to be limited) and Class 3 (solvents of low toxic potential) per the International Conference on Harmonization's (ICH) Guideline for Residual Solvents, July 1997). The use of mixtures of two or more of any of the aforementioned co-solvents is also included within the scope of the invention.

Class 2

Acetonitrile

Chlorobenzene

Cyclohexane

1,2-Dichloroethene

Dichloromethane

1,2-Dimethoxyethane

N,N-Dimethylformamide

N,N-Dimethylacetamide

1,4-Dioxane

2-Ethoxyethanol

Ethyleneglycol

Formamide

2-Methoxyethanol

Methylbutyl ketone

Methylcyclohexane

Nitromethane

Pyridine

Sulfolane

Tetralin

1,1,2-Trichloroethene

Class 3

1-Butanol

1-Pentanol

1-Propanol

2-Butanol

2-Methyl-1-propanol

2-Propanol (isopropyl alcohol)

3-Methyl-1-butanol

Acetone

Anisole

Butyl acetate

Cumene

Ethanol
Ethyl acetate
Ethyl ether
Ethyl formate
Isobutyl acetate
Isopropyl acetate
Methyl acetate
Methylethyl ketone
Methylisobutyl ketone
Propyl acetate
tert-Butylmethyl ether
Tetrahydrofuran

It is preferred, but not required, that the co-solvents are preheated before mixing with the primary solvent, preferably to a temperature below the temperature at which a substantial portion of the co-solvent would boil, most preferably to about 50°C. It is also preferred, but not required, that the co-solvent(s) is added gradually to the primary solvent(s).

Following mixing, the primary solvent(s)/co-solvent(s) mixture is then equilibrated at different temperatures in order to promote either a slow recrystallization or a fast recrystallization of Form I of 5-azacytidine, as described below.

By slow recrystallization is meant that the co-solvent/DMSO solution is allowed to equilibrate at a temperature in the range from about 0°C to about 40°C, preferably in the range of about 15°C to about 30°C, and most preferably at about ambient temperature. Slow recrystallization of Form I of 5-azacytidine is preferably performed using C_2-C_5 alcohols, aliphatic ketones, or alkyl cyanides as the co-solvent. More preferably, slow recrystallization is performed with Class 3 C_2-C_5 alcohols, Class 3 aliphatic ketones, or acetonitrile (Class 2). The most preferred Class 3 C_2-C_5 alchohols are ethanol, isopropyl alcohol, and 1-propanol, and the most preferred Class 3 aliphatic ketone is methylethyl ketone.

By fast recrystallization is meant that the co-solvent solution is allowed to equilibrate at a temperature of below 0°C, preferably below about -10°C, and most preferably at about -20°C.

Fast recrystallization of Form I of 5-azacytidine is preferably performed with a C_3 - C_5 alcohol (which term hereinafter refers to C_3-C_5 alcohols which are independently: branched or unbranched, substituted or unsubstituted) or an alkyl cyanide as the co-solvent. More preferably the C_3 - C_5 alcohol is a Class 3 solvent, and the alkyl cyanide is acetonitrile. The most preferred Class 3 C_3-C_5 alcohols are isopropyl alcohol (2-propanol) and 1-propanol.

Non-limiting examples of protocols for the recrystallization of Form I according to the methods described herein are provided in Examples 2 (slow recrystallization with DMSO as the primary solvent and ethanol, isopropyl alcohol, acetonitrile, or methylethyl ketone as the co-solvent) and 3 (fast recrystallization with DMSO as the primary solvent, and isopropyl alcohol or acetonitrile as the co-solvent) below.

Following recrystallization, the Form I of 5-azacytidine crystals may be isolated from the co-solvent mixture by any suitable method known in the art. Preferably, the Form I crystals are isolated using vacuum filtration through a suitable filter medium or by centrifugation.

Using the novel methods provided herein, it is possible for the first time to target Form I of 5-azacytidine as the drug substance reproducibly and robustly. In particular, isopropyl alcohol and acetonitrile reliably produce Form I independent of cooling rate (either slow recrystallization or fast recrystallization) and are preferred as the recrystallization co-solvents to recover Form I. Most preferably, Form I is isolated using isopropyl alcohol as the co-solvent since isopropyl alcohol carries a Class 3 risk classification (solvent of low toxic potential), whereas acetonitrile carries a Class 2 risk classification (solvent to be limited). The use of the DMSO/isopropyl alcohol system allows Form I of 5-azacytidine to be reliably recovered for the first time from solvents of low toxic potential without requiring control over the rate of recrystallation. In the most preferred embodiment, Form I of 5-azacytidine may be recovered simply by dissolving 5-azacytidine in DMSO (preferably heated to a temperature in the range of about 40°C to about 90°C prior to the addition of 5-azacytidine), adding isopropyl alcohol, and allowing the resulting solvent mixture to equilibrate at about ambient temperature.

In some embodiments of the invention, Form I of 5-azacytidine may be recovered from a primary solvent(s)/co-solvent(s) mixture by "seeding" with a small amount of Form I of 5-azacytidine either prior to, or during, the addition of the co-solvent(s). By seeding with Form

I, it is possible to expand the list of suitable co-solvents and co-solvent classes beyond those listed above. For example, it is known that recrystallization from the DMSO/methanol system produces either Form I, or a Form I/II mixed phase (see Example 1). If a small amount of Form I is added to the solution of 5-azacytidine in DMSO prior to addition of the methanol co-solvent, or is added during the addition of the methanol co-solvent, then Form I of 5-azacytidine may be reliably isolated.

By allowing the isolation of a single polymorphic form, one skilled in the art will appreciate that the present invention allows for the first time the production of 5-azacytidine drug substance with uniform and consistent properties from batch to batch, which properties include but are not limited to solubility and dissolution rate. In turn, this allows one to provide 5-azacytidine drug product (see below) which also has uniform and consistent properties from batch to batch.

Pharmaceutical Formulations

For the most effective administration of drug substance of the present invention, it is preferred to prepare a pharmaceutical formulation (also known as the "drug product" or "pharmaceutical composition") preferably in unit dose form, comprising one or more of the 5-azacytidine polymorphs of the present invention and one or more pharmaceutically acceptable carrier, diluent, or excipient. Most preferably, Form I 5-azacytidine prepared according to the methods provided herein is used to prepare the pharmaceutical formulation.

Such pharmaceutical formulation may, without being limited by the teachings set forth herein, include a solid form of the present invention which is blended with at least one pharmaceutically acceptable excipient, diluted by an excipient or enclosed within such a carrier that can be in the form of a capsule, sachet, tablet, buccal, lozenge, paper, or other container. When the excipient serves as a diluent, it may be a solid, semi-solid, or liquid material which acts as a vehicle, carrier, or medium for the 5-azacytidine polymorph(s). Thus, the formulations can be in the form of tablets, pills, powders, elixirs, suspensions, emulsions, solutions, syrups, capsules (such as, for example, soft and hard gelatin capsules), suppositories, sterile injectable solutions, and sterile packaged powders.

Examples of suitable excipients include, but are not limited to, starches, gum arabic, calcium silicate, microcrystalline cellulose, polyvinylpyrrolidone, cellulose, water, syrup, and

methyl cellulose. The formulations can additionally include lubricating agents such as, for example, talc, magnesium stearate and mineral oil; wetting agents; emulsifying and suspending agents; preserving agents such as methyl- and propyl- hydroxybenzoates; sweetening agents; or flavoring agents. Polyols, buffers, and inert fillers may also be used. Examples of polyols include, but are not limited to: mannitol, sorbitol, xylitol, sucrose, maltose, glucose, lactose, dextrose, and the like. Suitable buffers encompass, but are not limited to, phosphate, citrate, tartrate, succinate, and the like. Other inert fillers which may be used encompass those which are known in the art and are useful in the manufacture of various dosage forms. If desired, the solid pharmaceutical compositions may include other components such as bulling agents and/or granulating agents, and the like. The compositions of the invention can be formulated so as to provide quick, sustained, controlled, or delayed release of the drug substance after administration to the patient by employing procedures well known in the art.

In certain embodiments of the invention, the 5-azacytidine polymorph(s) may made into the form of dosage units for oral administration. The 5-azacytidine polymorph(s) may be mixed with a solid, pulverant carrier such as, for example, lactose, saccharose, sorbitol, mannitol, starch, amylopectin, cellulose derivatives or gelatin, as well as with an antifriction agent such as for example, magnesium stearate, calcium stearate, and polyethylene glycol waxes. The mixture is then pressed into tablets or filled into capsules. If coated tablets, capsules, or pulvules are desired, such tablets, capsules, or pulvules may be coated with a concentrated solution of sugar, which may contain gum arabic, gelatin, talc, titanium dioxide, or with a lacquer dissolved in the volatile organic solvent or mixture of solvents. To this coating, various dyes may be added in order to distinguish among tablets with different active compounds or with different amounts of the active compound present.

Soft gelatin capsules may be prepared in which capsules contain a mixture of the 5-azacytidine polymorph(s) and vegetable oil or non-aqueous, water miscible materials such as, for example, polyethylene glycol and the like. Hard gelatin capsules may contain granules or powder of the 5-azacytidine polymorph in combination with a solid, pulverulent carrier, such as, for example, lactose, saccharose, sorbitol, mannitol, potato starch, corn starch, amylopectin, cellulose derivatives, or gelatin.

Tablets for oral use are typically prepared in the following manner, although other techniques may be employed. The solid substances are gently ground or sieved to a desired particle size, and a binding agent is homogenized and suspended in a suitable solvent. The 5-azacytidine polymorph(s) and auxiliary agents are mixed with the binding agent solution. The resulting mixture is moistened to form a uniform suspension. The moistening typically causes the particles to aggregate slightly, and the resulting mass is gently pressed through a stainless steel sieve having a desired size. The layers of the mixture are then dried in controlled drying units for a pre-determined length of time to achieve a desired particle size and consistency. The granules of the dried mixture are gently sieved to remove any powder. To this mixture, disintegrating, anti-friction, and anti-adhesive agents are added. Finally, the mixture is pressed into tablets using a machine with the appropriate punches and dies to obtain the desired tablet size.

In the event that the above formulations are to be used for parenteral administration, such a formulation typically comprises sterile, aqueous and non-aqueous injection solutions comprising one or more 5-azacytidine polymorphs for which preparations are preferably isotonic with the blood of the intended recipient. These preparations may contain anti-oxidants, buffers, bacteriostats, and solute; which render the formulation isotonic with the blood of the intended recipient. Aqueous and non-aqueous suspensions may include suspending agents and thickening agents. The formulations may be present in unit-dose or multi-dose containers, for example, sealed ampules and vials. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules, and tablets of the kind previously described.

Liquid preparations for oral administration are prepared in the form of solutions, syrups, or suspensions with the latter two forms containing, for example, 5-azacytidine polymorph(s), sugar, and a mixture of ethanol, water, glycerol, and propylene glycol. If desired, such liquid preparations contain coloring agents, flavoring agents, and saccharin. Thickening agents such as carboxymethylcellulose may also be used.

As such, the pharmaceutical formulations of the present invention are preferably prepared in a unit dosage form, each dosage unit containing from about 5 mg to about 200 mg, more usually about 100 mg of the 5-azacytidine polymorph(s). In liquid form, dosage unit contains from about 5 to about 200 mg, more usually about 100 mg of the 5-azacytidine

polymorph(s). The term "unit dosage form" refers to physically discrete units suitable as unitary dosages for human subjects/patients or other mammals, each unit containing a predetermined quantity of the 5-azacytidine polymorph calculated to produce the desired therapeutic effect, in association with preferably, at least one pharmaceutically acceptable carrier, diluent, or excipient.

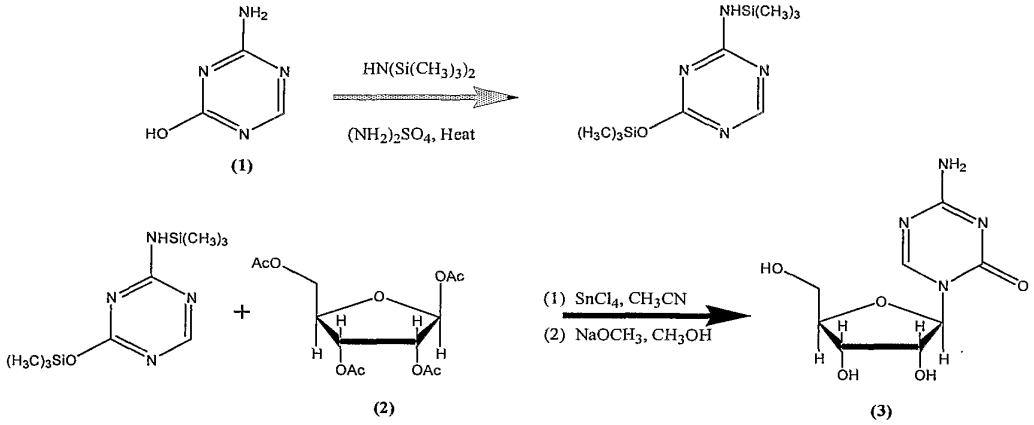
The following examples are provided for illustrative purposes only, and are not to be construed as limiting the scope of the claims in any way.

Examples

Example 1

Prior Art Procedure for Synthesis and Recrystallization of 5-azacytidine Drug Substance

5-azacytidine may be synthesized using commercially available 5-azacytosine and 1,2,3,5-tetra-O-acetyl-β-D-ribofuranose (RTA) according to the following pathway:



The crude synthesis product is dissolved in DMSO (preheated to about 90°C), and then methanol is added to the DMSO solution. The co-solvent mixture is equilibrated at approximately -20°C to allow 5-azacytidine crystal formation. The product is collected by vacuum filtration and allowed to air dry.

Example 2

Form I of 5-azacytidine: Slow Recrystallization of 5-azacytidine from Co-Solvent Systems

Approximately 250 mg of 5-azacytidine was dissolved with approximately 5 ml of dimethyl sulfoxide (DMSO), preheated to approximately 90 °C, in separate 100-mL beakers. The solids were allowed to dissolve to a clear solution. Approximately 45 mL of ethanol, isopropyl alcohol, acetonitrile, or methyl ethyl ketone co-solvent, preheated to approximately 50 °C, was added to the solution and the resultant solution was mixed. The solution was covered and allowed to equilibrate at ambient conditions. The product was collected by vacuum filtration using a Buchner funnel.

Example 3

Form I of 5-azacytidine: Fast Recrystallization of 5-azacytidine from Co-Solvent Systems

Approximately 250 mg of 5-azacytidine was dissolved with approximately 5 mL of DMSO, preheated to approximately 90 °C, in separate 100-ml beakers. The solids were allowed to dissolve to a clear solution. Approximately 45 mL of isopropyl alcohol or acetonitrile co-solvent, preheated to approximately 50 °C, was added to the solution and the resultant solution was mixed. The solution was covered and placed in a freezer to equilibrate at approximately -20°C to allow crystal formation. Solutions were removed from the freezer after crystal formation. The product was collected by vacuum filtration using a Buchner funnel.

Example 4

X-Ray Powder Diffraction of Recrystallized 5-azacytidine

X-ray powder diffraction (XRPD) patterns for each sample were obtained on a Scintag XDS 2000 or a Scintag X_2 θ/θ diffractometer operating with copper radiation at 45 kV and 40 mA using a Kevex Psi Peltier-cooled silicon detector or a Thermo ARL Peltier-cooled solid state detector. Source slits of 2 or 4 mm and detector slits of 0.5 or 0.3 mm were used for data collection. Recrystallized material was gently milled for approximately one minute using an agate mortar and pestle. Samples were placed in a stainless steel or silicon sample holder and leveled using a glass microscope slide. Powder diffraction patterns of the samples were obtained from 2 to 42° 2θ at 1°/minute. Calibration of the X_2 diffractometer is verified annually using a silicon powder standard.

XRPD performed according to this method revealed that the Form I of 5-azacytidine was isolated in Example 2 by slow recrystallization using either ethanol, isopropyl alcohol, acetonitrile, or methyl ethyl ketone as the co-solvent, and in Example 3 by fast recrystallization using isopropyl alcohol or acetonitrile as the co-solvent. The results indicate that Form I of 5-azacytidine may be reliably recovered from the DMSO/isopropyl alcohol and DMSO/acetonitrile solvent systems without control of the rate of recrystallization.

What is claimed is:

1. A method for isolating crystalline Form I of 5-azacytidine substantially free of other forms, the method comprising:

recrystallizing 5-azacytidine from a solvent mixture comprising at least one primary solvent and at least one co-solvent selected from the group consisting of:

1,1,2-Trichloroethene
1,2-Dichloroethene
1,2-Dimethoxyethane
1,4-Dioxane
1-Butanol
1-Pentanol
1-Propanol
2-Butanol
2-Ethoxyethanol
2-Methoxyethanol
2-Methyl-1-propanol
2-Propanol (isopropyl alcohol)
3-Methyl-1-butanol
Acetone
Acetonitrile
Anisole
Butyl acetate
Chlorobenzene
Cumene
Cyclohexane
Dichloromethane
Ethanol
Ethyl acetate
Ethyl ether
Ethyl formate
Ethyleneglycol
Formamide
Isobutyl acetate

Isopropyl acetate
Methyl acetate
Methylbutyl ketone
Methylcyclohexane
Methylethyl ketone
Methylisobutyl ketone
N,N-Dimethylacetamide
N,N-Dimethylformamide
Nitromethane
Propyl acetate
Pyridine
Sulfolane
tert-Butylmethyl ether
Tetrahydrofuran, and
Tetralin

by cooling said solvent mixture from a temperature selected to allow said 5-azacytidine to dissolve completely to about ambient temperature; and
isolating the recrystallized 5-azacytidine.

2. The method of claim 1 wherein said primary solvent is a polar solvent.

3. The method of claim 2 wherein said polar solvent is a polar aprotic solvent.

4. The method of claim 3 wherein said polar aprotic solvent is selected from the group consisting of dimethylsulfoxide, dimethylformamide, dimethylacetamide, and N-methylpyrrolidinone.

5. The method of claim 4 wherein said polar aprotic solvent is dimethylsulfoxide.

6. The method of claim 1 wherein said co-solvent is acetonitrile.

7. The method of claim 1 wherein said co-solvent is ethanol.

8. The method of claim 1 wherein said co-solvent is methylethyl ketone.

9. The method of claim 1 wherein said co-solvent is 2-propanol (isopropyl alcohol).

10. The method of claim 1 wherein said co-solvent is 1-propanol.

11. A method for isolating crystalline Form I of 5-azacytidine substantially free of other forms, the method comprising:

recrystallizing 5-azacytidine from a solvent mixture comprising at least one primary solvent and at least one co-solvent selected from the group consisting of C_2-C_5 alcohols, aliphatic ketones, and alkyl cyanides, by cooling said solvent mixture from a temperature selected to allow said 5-azacytidine to dissolve completely to about ambient temperature; and

isolating the recrystallized 5-azacytidine.

12. The method of claim 11 wherein said primary solvent is a polar solvent.

13. The method of claim 12 wherein said polar solvent is a polar aprotic solvent.

14. The method of claim 13 wherein said polar aprotic solvent is selected from the group consisting of dimethylsulfoxide, dimethylformamide, dimethylacetamide, and N-methylpyrrolidinone.

15. The method of claim 14 wherein said polar aprotic solvent is dimethylsulfoxide.

16. The method of claim 11 wherein said co-solvent is acetonitrile.

17. The method of claim 11 wherein said co-solvent is ethanol.

18. The method of claim 11 wherein said co-solvent is methylethyl ketone.

19. The method of claim 11 wherein said co-solvent is 2-propanol (isopropyl alcohol).

20. The method of claim 11 wherein said co-solvent is 1-propanol.

21. A method for isolating crystalline Form I 5-azacytidine substantially free of other forms, the method comprising:

recrystallizing 5-azacytidine from a solvent mixture comprising at least one primary solvent and at least one co-solvent selected from the group consisting of:

1,1,2-Trichloroethene
1,2-Dichloroethene
1,2-Dimethoxyethane
1,4-Dioxane
1-Butanol
1-Pentanol
1-Propanol
2-Butanol
2-Ethoxyethanol
2-Methoxyethanol
2-Methyl-1-propanol
2-Propanol
3-Methyl-1-butanol
Acetone
Acetonitrile
Anisole
Butyl acetate
Chlorobenzene
Cumene
Cyclohexane
Dichloromethane
Ethyl acetate
Ethyl ether
Ethyl formate
Ethyleneglycol
Formamide
Isobutyl acetate
Isopropyl acetate

Methyl acetate
Methylbutyl ketone
Methylcyclohexane
Methylisobutyl ketone
N,N-Dimethylacetamide
N,N-Dimethylformamide
Nitromethane
Propyl acetate
Pyridine
Sulfolane
tert-Butylmethyl ether
Tetrahydrofuran, and
Tetralin

by cooling said solution from a temperature selected to allow said 5-azacytidine to dissolve completely to about -20°C ; and
isolating the recrystallized 5-azacytidine.

22. The method of claim 21 wherein said primary solvent is a polar solvent.

23. The method of claim 22 wherein said polar solvent is a polar aprotic solvent.

24. The method of claim 23 wherein said polar aprotic solvent is selected from the group consisting of dimethylsulfoxide, dimethylformamide, dimethylacetamide, and N-methylpyrrolidinone.

25. The method of claim 24 wherein said polar aprotic solvent is dimethylsulfoxide.

26. The method of claim 21 wherein said co-solvent is acetonitrile.

27. The method of claim 21 wherein said co-solvent is 2-propanol (isopropyl alcohol).

28. The method of claim 21 wherein said co-solvent is 1-propanol.

29. A method for isolating crystalline Form I of 5-azacytidine substantially free of other forms, the method comprising:

recrystallizing 5-azacytidine from a solvent mixture comprising at least one primary solvent and at least one co-solvent selected from the group consisting of C_3 - C_5 alcohols and alkyl cyanides by cooling said solution from a temperature selected to allow said 5-azacytidine to dissolve completely to about -20°C ; and

isolating the recrystallized 5-azacytidine.

30. The method of claim 29 wherein said primary solvent is a polar solvent.

31. The method of claim 30 wherein said polar solvent is a polar aprotic solvent.

32. The method of claim 31 wherein said polar aprotic solvent is selected from the group consisting of dimethylsulfoxide, dimethylformamide, dimethylacetamide, and N-methylpyrrolidinone.

33. The method of claim 32 wherein said polar aprotic solvent is dimethylsulfoxide.

34. The method of claim 29 wherein said co-solvent is acetonitrile.

35. The method of claim 29 wherein said co-solvent is 2-propanol (isopropyl alcohol).

36. The method of claim 29 wherein said co-solvent is 1-propanol.

37. A method for isolating crystalline Form I of 5-azacytidine substantially free of other forms, the method comprising:

recrystallizing 5-azacytidine from a solvent mixture comprising dimethylsulfoxide and isopropyl alcohol; and

isolating the recrystallized 5-azacytidine.

38. A method for isolating crystalline Form I of 5-azacytidine substantially free of other forms, the method comprising:

recrystallizing 5-azacytidine from a solvent mixture comprising dimethylsulfoxide and 1-propanol; and

isolating the recrystallized 5-azacytidine.

39. A method for isolating crystalline Form I of 5-azacytidine substantially free of other forms, the method comprising:

recrystallizing 5-azacytidine from a solvent mixture comprising dimethylsulfoxide and methanol by seeding said solvent mixture with Form I of 5-azacytidine; and isolating the recrystallized 5-azacytidine.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
24 August 2006 (24.08.2006)

PCT

(10) International Publication Number
WO 2006/089290 A1

(51) International Patent Classification:
A61K 31/337 (2006.01) *A61K 31/7068* (2006.01)
A61K 31/555 (2006.01) *A61P 35/00* (2006.01)
A61K 38/22 (2006.01)

(21) International Application Number:
PCT/US2006/006167

(22) International Filing Date:
21 February 2006 (21.02.2006)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/654,245 18 February 2005 (18.02.2005) US

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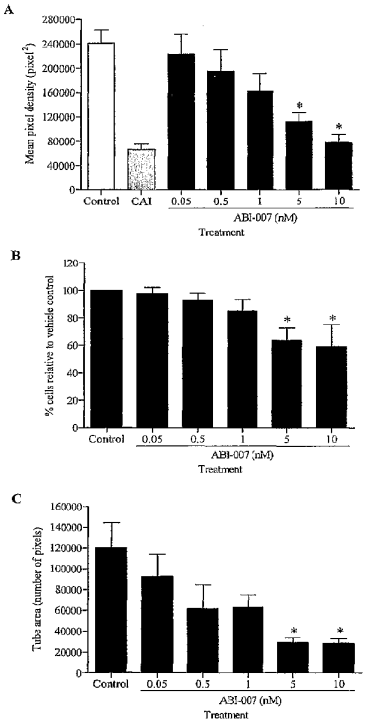
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM,

[Continued on next page]

(54) Title: COMBINATIONS AND MODES OF ADMINISTRATION OF THERAPEUTIC AGENTS AND COMBINATION THERAPY



(57) Abstract: The present invention provides combination therapy methods of treating proliferative diseases (such as cancer) comprising a first therapy comprising administering to an individual an effective amount of a taxane in a nanoparticle composition, and a second therapy which may include, for example, radiation, surgery, administration of chemotherapeutic agents, or combinations thereof. Also provided are methods of administering to an individual a drug taxane in a nanoparticle composition based on a metronomic dosing regime.

WO 2006/089290 A1

ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

COMBINATIONS AND MODES OF ADMINISTRATION OF THERAPEUTIC AGENTS AND COMBINATION THERAPY

RELATED APPLICATIONS

[0001] This application claims priority benefit to provisional application 60/654,245, filed on February 18, 2005, which is incorporated by reference herein in its entirety.

TECHNICAL FIELD

[0002] The present invention relates to methods and compositions for the treatment of proliferative diseases comprising the administration of a combination of a taxane and at least one other and other therapeutic agents, as well as other treatment modalities useful in the treatment of proliferative diseases. In particular, the invention relates to the use of nanoparticles comprising paclitaxel and albumin (such as Abraxane™) in combination with other chemotherapeutic agents or radiation, which may be used for the treatment of cancer.

BACKGROUND

[0003] The failure of a significant number of tumors to respond to drug and/or radiation therapy is a serious problem in the treatment of cancer. In fact, this is one of the main reasons why many of the most prevalent forms of human cancer still resist effective chemotherapeutic intervention, despite certain advances in the field of chemotherapy.

[0004] Cancer is now primarily treated with one or a combination of three types of therapies: surgery, radiation, and chemotherapy. Surgery is a traditional approach in which all or part of a tumor is removed from the body. Surgery generally is only effective for treating the earlier stages of cancer. While surgery is sometimes effective in removing tumors located at certain sites, for example, in the breast, colon, and skin, it cannot be used in the treatment of tumors located in other areas, inaccessible to surgeons, nor in the treatment of disseminated neoplastic conditions such as leukemia. For more than 50% of cancer individuals, by the time they are diagnosed they are no longer candidates for effective surgical treatment. Surgical procedures may increase tumor metastases through blood circulation during surgery. Most of cancer individuals do not die from the cancer at the time of diagnosis or surgery, but rather die from the metastasis and the recurrence of the cancer.

[0005] Other therapies are also often ineffective. Radiation therapy is only effective for individuals who present with clinically localized disease at early and middle

stages of cancer, and is not effective for the late stages of cancer with metastasis. Radiation is generally applied to a defined area of the subject's body which contains abnormal proliferative tissue, in order to maximize the dose absorbed by the abnormal tissue and minimize the dose absorbed by the nearby normal tissue. However, it is difficult (if not impossible) to selectively administer therapeutic radiation to the abnormal tissue. Thus, normal tissue proximate to the abnormal tissue is also exposed to potentially damaging doses of radiation throughout the course of treatment. There are also some treatments that require exposure of the subject's entire body to the radiation, in a procedure called "total body irradiation", or "TBI." The efficacy of radiotherapeutic techniques in destroying abnormal proliferative cells is therefore balanced by associated cytotoxic effects on nearby normal cells. Because of this, radiotherapy techniques have an inherently narrow therapeutic index which results in the inadequate treatment of most tumors. Even the best radiotherapeutic techniques may result in incomplete tumor reduction, tumor recurrence, increasing tumor burden, and induction of radiation resistant tumors.

[0006] Chemotherapy involves the disruption of cell replication or cell metabolism. Chemotherapy can be effective, but there are severe side effects, e.g., vomiting, low white blood cells (WBC), loss of hair, loss of weight and other toxic effects. Because of the extremely toxic side effects, many cancer individuals cannot successfully finish a complete chemotherapy regime. Chemotherapy-induced side effects significantly impact the quality of life of the individual and may dramatically influence individual compliance with treatment. Additionally, adverse side effects associated with chemotherapeutic agents are generally the major dose-limiting toxicity (DLT) in the administration of these drugs. For example, mucositis is one of the major dose limiting toxicity for several anticancer agents, including the antimetabolite cytotoxic agents 5-FU, methotrexate, and antitumor antibiotics, such as doxorubicin. Many of these chemotherapy-induced side effects if severe may lead to hospitalization, or require treatment with analgesics for the treatment of pain. Some cancer individuals die from the chemotherapy due to poor tolerance to the chemotherapy. The extreme side effects of anticancer drugs are caused by the poor target specificity of such drugs. The drugs circulate through most normal organs of individuals as well as intended target tumors. The poor target specificity that causes side effects also decreases the efficacy of chemotherapy because only a fraction of the drugs is correctly targeted. The efficacy of chemotherapy is further decreased by poor retention of the anti-cancer drugs within the target tumors.

[0007] Due to the severity and breadth of neoplasm, tumor and cancer, there is a great need for effective treatments of such diseases or disorders that overcome the shortcomings of surgery, chemotherapy, and radiation treatment.

Problems of Chemotherapeutic Agents

[0008] The drug resistance problem is a reason for the added importance of combination chemotherapy, as the therapy both has to avoid the emergence of resistant cells and to kill pre-existing cells which are already drug resistant.

[0009] Drug resistance is the name given to the circumstance when a disease does not respond to a treatment drug or drugs. Drug resistance can be either intrinsic, which means the disease has never been responsive to the drug or drugs, or it can be acquired, which means the disease ceases responding to a drug or drugs that the disease had previously been responsive to. Multidrug resistance (MDR) is a specific type of drug resistance that is characterized by cross-resistance of a disease to more than one functionally and/or structurally unrelated drugs. Multidrug resistance in the field of cancer is discussed in greater detail in "Detoxification Mechanisms and Tumor Cell Resistance to Anticancer Drugs," by Kuzmich and Tew, particularly section VII "The Multidrug-Resistant Phenotype (MDR)," Medical Research Reviews, Vol. 11, No. 2, 185-217, (Section VII is at pp. 208-213) (1991); and in "Multidrug Resistance and Chemosensitization: Therapeutic Implications for Cancer Chemotherapy," by Georges, Sharom and Ling, Advances in Pharmacology, Vol. 21, 185-220 (1990).

[0010] One form of multi-drug resistance (MDR) is mediated by a membrane bound 170-180 kD energy-dependent efflux pump designated as P-glycoprotein (P-gp). P-glycoprotein has been shown to play a major role in the intrinsic and acquired resistance of a number of human tumors against hydrophobic, natural product drugs. Drugs that act as substrates for and are consequently detoxified by P-gp include the vinca alkaloids (vincristine and vinblastine), anthracyclines (Adriamycin), and epipodophyllotoxins (etoposide). While P-gp associated MDR is a major determinant in tumor cell resistance to chemotherapeutic agents, it is clear that the phenomenon of MDR is multifactorial and involves a number of different mechanisms.

[0011] A major complication of cancer chemotherapy and of antiviral chemotherapy is damage to bone marrow cells or suppression of their function. Specifically, chemotherapy damages or destroys hematopoietic precursor cells, primarily found in the bone marrow and spleen, impairing the production of new blood cells

(granulocytes, lymphocytes, erythrocytes, monocytes, platelets, etc.). Treatment of cancer individuals with 5-fluorouracil, for example, reduces the number of leukocytes (lymphocytes and/or granulocytes), and can result in enhanced susceptibility of the individuals to infection. Many cancer individuals die of infection or other consequences of hematopoietic failure subsequent to chemotherapy. Chemotherapeutic agents can also result in subnormal formation of platelets which produces a propensity toward hemorrhage. Inhibition of erythrocyte production can result in anemia. For some cancer individuals, the risk of damage to the hematopoietic system or other important tissues frequently limits the opportunity for chemotherapy dose escalation of chemotherapy agents high enough to provide good antitumor or antiviral efficacy. Repeated or high dose cycles of chemotherapy may be responsible for severe stem cell depletion leading to serious long-term hematopoietic sequelea and marrow exhaustion.

[0012] Prevention of, or protection from, the side effects of chemotherapy would be a great benefit to cancer individuals. For life-threatening side effects, efforts have concentrated on altering the dose and schedules of the chemotherapeutic agent to reduce the side effects. Other options are becoming available, such as the use of granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage-CSF (GM-CSF), epidermal growth factor (EGF), interleukin 11, erythropoietin, thrombopoietin, megakaryocyte development and growth factor, pixykines, stem cell factor, FLT-ligand, as well as interleukins 1, 3, 6, and 7, to increase the number of normal cells in various tissues before the start of chemotherapy (See Jimenez and Yunis, Cancer Research 52:413-415; 1992). The mechanisms of protection by these factors, while not fully understood, are most likely associated with an increase in the number of normal critical target cells before treatment with cytotoxic agents, and not with increased survival of cells following chemotherapy.

Chemotherapeutic Targeting For Tumor Treatment

[0013] Both the growth and metastasis of solid tumors are angiogenesis-dependent (Folkman, J. *Cancer* Res., 46, 467-73 (1986); Folkman, J. Nat. *Cancer* Inst., 82, 4-6 (1989); Folkman et al., "Tumor Angiogenesis," Chapter 10, pp. 206-32, in The Molecular Basis of *Cancer,* Mendelsohn et al., eds. (W. B. Saunders, 1995)). It has been shown, for example, that tumors which enlarge to greater than 2 mm in diameter must obtain their own blood supply and do so by inducing the growth of new capillary blood vessels. After these new blood vessels become embedded in the tumor, they provide nutrients and growth factors essential for tumor growth as well as a means for tumor cells to enter the circulation

and metastasize to distant sites, such as liver, lung or bone (Weidner, New Eng. J. Med., 324(1), 1-8 (1991)). When used as drugs in tumor-bearing animals, natural inhibitors of angiogenesis can prevent the growth of small tumors (O'Reilly et al., O'Reilly et al., Cell, 79, 315-28 (1994)). Indeed, in some protocols, the application of such inhibitors leads to tumor regression and dormancy even after cessation of treatment (O'Reilly et al., Cell, 88, 277-85 (1997)). Moreover, supplying inhibitors of angiogenesis to certain tumors can potentiate their response to other therapeutic regimes (e.g., chemotherapy) (see, e.g., Teischer et al., Int. J. *Cancer*, 57, 920-25 (1994)).

[0014] Protein tyrosine kinases catalyze the phosphorylation of specific tyrosyl residues in various proteins involved in the regulation of cell growth and differentiation (A. F. Wilks, Progress in Growth Factor Research, 1990, 2, 97-111; S. A. Courtneidge, Dev. Supp.l, 1993, 57-64; J. A. Cooper, Semin. Cell Biol., 1994, 5(6), 377-387; R. F. Paulson, Semin. Immunol., 1995, 7(4), 267-277; A. C. Chan, Curr. Opin. Immunol., 1996, 8(3), 394-401). Protein tyrosine kinases can be broadly classified as receptor (e.g. EGFr, c-erbB-2, c-met, tie-2, PDGFr, FGFr) or non-receptor (e.g. c-src, Ick, Zap70) kinases. Inappropriate or uncontrolled activation of many of these kinases, i.e. aberrant protein tyrosine kinase activity, for example by over-expression or mutation, has been shown to result in uncontrolled cell growth. For example, elevated epidermal growth factor receptor (EGFR) activity has been implicated in non-small cell lung, bladder and head and neck cancers, and increased c-erbB-2 activity in breast, ovarian, gastric and pancreatic cancers. Thus, inhibition of protein tyrosine kinases should be useful as a treatment for tumors such as those outlined above.

[0015] Growth factors are substances that induce cell proliferation, typically by binding to specific receptors on cell surfaces. Epidermal growth factor (EGF) induces proliferation of a variety of cells *in vivo*, and is required for the growth of most cultured cells. The EGF receptor is a 170-180 kD membrane-spanning glycoprotein, which is detectable on a wide variety of cell types. The extracellular N-terminal domain of the receptor is highly glycosylated and binds EGF antibodies that selectively bind to EGFR. Agents that competitively bind to EGFR have been used to treat certain types of cancer, since many tumors of mesodermal and ectodermal origin overexpress the EGF receptor. For example, the EGF receptor has been shown to be overexpressed in many gliomas, squamous cell carcinomas, breast carcinomas, melanomas, invasive bladder carcinomas and esophageal cancers. Attempts to exploit the EGFR system for anti-tumor therapy have generally involved the use of monoclonal antibodies against the EGFR. In addition, studies

with primary human mammary tumors have shown a correlation between high EGFR expression and the presence of metastases, higher rates of proliferation, and shorter individual survival.

[0016] Herlyn et al., in U.S. Patent 5,470,571, disclose the use of radiolabeled Mab 425 for treating gliomas that express EGFR. Herlyn et al. report that anti-EGFR antibodies may either stimulate or inhibit cancer cell growth and proliferation. Other monoclonal antibodies having specificity for EGFR, either alone or conjugated to a cytotoxic compound, have been reported as being effective for treating certain types of cancer. Bendig et al, in U.S. Patent 5,558,864, disclose therapeutic anti-EGFR Mab's for competitively binding to EGFR. Heimbrook et al., in U.S. Patent 5,690,928, disclose the use of EGF fused to a *Pseudomonas* species-derived endotoxin for the treatment of bladder cancer. Brown et al., in U.S. Patent 5,859,018, disclose a method for treating diseases characterized by cellular hyperproliferation mediated by, inter alia, EGF.

Chemotherapeutic Modes of Administration

[0017] People diagnosed as having cancer are frequently treated with single or multiple chemotherapeutic agents to kill cancer cells at the primary tumor site or at distant sites to where cancer has metastasized. Chemotherapy treatment is typically given either in a single or in several large doses or over variable times of weeks to months. However, repeated or high dose cycles of chemotherapy may be responsible for increased toxicities and severe side effects.

[0018] New studies suggest that metronomic chemotherapy, the low-dose and frequent administration of cytotoxic agents without prolonged drug-free breaks, targets activated endothelial cells in the tumor vasculature. A number of preclinical studies have demonstrated superior anti-tumor efficacy, potent antiangiogenic effects, and reduced toxicity and side effects (e.g., myelosuppression) of metronomic regimes compared to maximum tolerated dose (MTD) counterparts (Bocci, et al., Cancer Res, 62:6938-6943, (2002); Bocci, et al., PNAS, vol, 100(22):12917-12922, (2003); and Bertolini, et al., Cancer Res, 63(15):4342-4346, (2003)). It remains unclear whether all chemotherapeutic drugs exert similar effects or whether some are better suited for such regimes than others. Nevertheless, metronomic chemotherapy appears to be effective in overcoming some of the major shortcomings associated with chemotherapy.

Chemotherapeutic Agents

[0019] Paclitaxel has been shown to have significant antineoplastic and anticancer effects in drug-refractory ovarian cancer and has shown excellent antitumor activity in a wide variety of tumor models, and also inhibits angiogenesis when used at very low doses (Grant et al., Int. J. Cancer, 2003). The poor aqueous solubility of paclitaxel, however, presents a problem for human administration. Indeed, the delivery of drugs that are inherently insoluble or poorly soluble in an aqueous medium can be seriously impaired if oral delivery is not effective. Accordingly, currently used paclitaxel formulations (e.g., Taxol®) require a Cremophor® to solubilize the drug. The presence of Cremophor® in this formulation has been linked to severe hypersensitivity reactions in animals (Lorenz et al., *Agents Actions* 7:63-67 (1987)) and humans (Weiss et al., *J. Clin. Oncol.* 8:1263-68 (1990)) and consequently requires premedication of individuals with corticosteroids (dexamethasone) and antihistamines. It was also reported that clinically relevant concentrations of the formulation vehicle Cremophor® EL in Taxol® nullify the antiangiogenic activity of paclitaxel, suggesting that this agent or other anticancer drugs formulated in Cremophor® EL may need to be used at much higher doses than anticipated to achieve effective metronomic chemotherapy (Ng et al., *Cancer Res.*, 64:821-824 (2004)). As such, the advantage of the lack of undesirable side effects associated with low-dose paclitaxel regimes vs. conventional MTD chemotherapy may be compromised. See also U.S. Patent Pub. No. 2004/0143004; WO00/64437.

Abraxane™ is a Cremophor® EL-free nanoparticle albumin-bound paclitaxel

[0020] Preclinical models have shown significant improvement in the safety and efficacy of Abraxane™ compared with Taxol® (Desai et al., EORTC-NCI-AACR, 2004) and in individuals with metastatic breast cancer (O'Shaughnessy et al., San Antonio Breast Cancer Symposium, Abstract #1122, Dec. 2003). This is possibly due to the absence of surfactants (e.g., Cremophor® or Tween® 80, used in Taxol® and Taxotere®, respectively) in Abraxane™, and/or preferential utilization of an albumin-based transport mechanism utilizing gp60/caveolae on microvascular endothelial cells (Desai et al., EORTC-NCI-AACR, 2004). In addition, both Cremophor® and Tween® 80 have been shown to strongly inhibit the binding of paclitaxel to albumin, possibly affecting albumin based transport (Desai et al., EORTC-NCI-AACR, 2004).

[0021] IDN5109 (Ortataxel) is a new taxane, currently in phase II, selected for its lack of cross-resistance in tumor cell lines expressing the multidrug resistant phenotype

(MDR/Pgp) and inhibition of P-glycoprotein (Pgp) (Minderman; *Cancer Chemother. Pharmacol.* 2004; 53:363-9). Due to its hydrophobicity, IDN5109 is currently formulated in the surfactant Tween® 80 (same vehicle as Taxotere®). Removal of surfactants from taxane formulations e.g., in the case of nanoparticle albumin-bound paclitaxel (Abraxane™) showed improvements in safety and efficacy over their surfactant containing counterparts (O'Shaughnessy et al., San Antonio Breast Cancer Symposium, Abstract #1122, Dec. 2003). Tween® 80 also strongly inhibited the binding of the taxane, paclitaxel, to albumin, possibly compromising albumin based drug transport via the gp60 receptor on microvessel endothelial cells (Desai et al., EORTC-NCI-AACR, 2004).

[0022] The antitumor activity of colchicine, which is the major alkaloid of the autumn crocus, *Colchicum autumnale*, and the African climbing lily, *Gloriosa superba*, was first reported at the beginning of the 20th century. The elucidation of its structure was finally completed from X-ray studies and a number of total syntheses (see Shiau et al., *J. Pharm. Sci.* 1978, 67(3) 394-397). Colchicine is thought to be a mitotic poison, particularly in tyhmic, intestinal, and hermatopoietic cells, which acts as a spindle poison and blocks the kinesis. Its effect on the mitotic spindle is thought to represent a special case of its effects on various organized, labile, fibrillar systems concerned with structure and movement.

[0023] Thiocolchicine dimer IDN5404 was selected for its activity in human ovarian subline resistant to cisplatin and topotecan A2780-CIS and A2780-TOP. This effect was related to dual mechanisms of action, i.e., microtubule activity as in Vinca alkaloids and a topoisomerase I inhibitory effect different from camptothecin. (Raspaglio, *Biochemical Pharmacology* 69:113-121 (2005)).

[0024] It has been found that nanoparticle compositions of a taxane (such as albumin bound paclitaxel (Abraxane™)) have significantly lower toxicities than other taxanes like Taxol® and Taxotere® with significantly improved outcomes in both safety and efficacy.

[0025] Combination chemotherapy, e.g., combining one or more chemotherapeutic agents or other modes of treatment, e.g., combining for example, chemotherapy with radiation or surgery and chemotherapy, have been found to be more successful than single agent chemotherapeutics or individual modes of treatment respectively.

[0026] Other references include U.S. Pub. No. 2006/0013819; U.S. Pub. No. 2006/0003931; WO05/117986; WO05/117978; and WO05/000900.

[0027] More effective treatments for proliferative diseases, especially cancer, are needed.

[0028] The disclosures of all publications, patents, patent applications and published patent applications referred to herein are hereby incorporated herein by reference in their entirety.

BRIEF SUMMARY OF THE INVENTION

[0029] The present invention provides methods for the treatment of proliferative diseases such as cancer. The invention provides combination therapy methods of treating proliferative diseases (such as cancer), comprising a) a first therapy comprising administering to an individual an effective amount of a composition comprising nanoparticles comprising a taxane (such as paclitaxel) and a carrier protein (such as albumin) and b) a second therapy, such as chemotherapy, radiation therapy, surgery, or combinations thereof. In another aspect, there are provided methods of administering to an individual a composition comprising nanoparticles comprising a taxane (such as paclitaxel) and a carrier protein (such as albumin) based on a metronomic dosing regime.

[0030] In some embodiments, the invention provides a method of treating a proliferative disease (such as cancer) in an individual comprising administering to the individual a) an effective amount of a composition comprising nanoparticles comprising a taxane and a carrier protein (such as albumin), and b) an effective amount of at least one other chemotherapeutic agent. In some embodiments, the invention provides a method of treating a proliferative disease (such as cancer) in an individual comprising administering to the individual a) an effective amount of a composition comprising nanoparticles comprising paclitaxel and an albumin (such as Abraxane™), and b) an effective amount of at least one other chemotherapeutic agent. In some embodiments, the chemotherapeutic agent is any of (and in some embodiments selected from the group consisting of) antimetabolites (including nucleoside analogs), platinum-based agents, alkylating agents, tyrosine kinase inhibitors, anthracycline antibiotics, vinca alkloids, proteasome inhibitors, macrolides, and topoisomerase inhibitors. In some embodiments, the chemotherapeutic agent is a platinum-based agent, such as carboplatin.

[0031] In some embodiments, the composition comprising nanoparticles (also referred to as "nanoparticle composition") and the chemotherapeutic agent are administered simultaneously, either in the same composition or in separate compositions. In some embodiments, the nanoparticle composition and the chemotherapeutic agent are

administered sequentially, i.e., the nanoparticle composition is administered either prior to or after the administration of the chemotherapeutic agent. In some embodiments, the administration of the nanoparticle composition and the chemotherapeutic agent are concurrent, i.e., the administration period of the nanoparticle composition and that of the chemotherapeutic agent overlap with each other. In some embodiments, the administration of the nanoparticle composition and the chemotherapeutic agent are non-concurrent. For example, in some embodiments, the administration of the nanoparticle composition is terminated before the chemotherapeutic agent is administered. In some embodiments, the administration of the chemotherapeutic agent is terminated before the nanoparticle composition is administered.

[0032] In some embodiments, the first therapy taxane is nano-particle albumin bound paxlitaxel, described, for example, in U.S. Patent 6,566,405, and commercially available under the tradename Abraxane™. In addition, the first therapy taxane is also considered to be nanoparticle albumin bound docetaxel described for example in U.S. Patent Application Publication 2005/0004002A1.

[0033] In another aspect, there is provided a method of treating a proliferative disease (such as cancer) in an individual comprising a) a first therapy comprising administering to the individual a composition comprising nanoparticles comprising a taxane and a carrier protein (such as albumin), and b) a second therapy comprising radiation therapy, surgery, or combinations thereof. In some embodiments, there is provided a method of treating a proliferative disease (such as cancer) in an individual comprising a) a first therapy comprising administering to the individual a composition comprising nanoparticles comprising paclitaxel and an albumin (such as Abraxane™), and b) a second therapy comprising radiation therapy, surgery, or combinations thereof. In some embodiments, the second therapy is radiation therapy. In some embodiments, the second therapy is surgery. In some embodiments, the first therapy is carried out prior to the second therapy. In some embodiments, the first therapy is carried out after the second therapy.

[0034] In another aspect, the method comprises administering to a mammal having a proliferative disease (such as cancer) a combination therapy comprising a first therapy comprising a taxane and a second therapy selected from the group consisting of chemotherapeutic agent and radiation or combinations thereof. The combination therapy may be administered in any of a variety of ways such as sequentially or simultaneously, and if sequential, the taxane may be administered before or after the second therapy

although it is preferred that the first therapy comprising a taxane is administered first. It will also be understood that the second therapy can include more than one chemotherapeutic agent.

[0035] The present invention also provides metronomic therapy regimes. In some embodiments, there is provided a method of administering a composition comprising nanoparticles comprising a taxane and a carrier protein (such as albumin), wherein the nanoparticle composition is administered over a period of at least one month, wherein the interval between each administration is no more than about a week, and wherein the dose of taxane at each administration is about 0.25% to about 25% of its maximum tolerated dose following a traditional dosing regime. In some embodiments, there is provided a method of administering a composition comprising nanoparticles comprising paclitaxel and an albumin (such as Abraxane^{TM}), wherein the nanoparticle composition is administered over a period of at least one month, wherein the interval between each administration is no more than about a week, and wherein the dose of paclitaxel at each administration is about 0.25% to about 25% of its maximum tolerated dose following a traditional dosing regime. In some embodiments, the dose of the taxane (such as paclitaxel, for example Abraxane^{TM}) per administration is less than about any of 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 18%, 20%, 22%, 24%, or 25% of the maximum tolerated dose. In some embodiments, the nanoparticle composition is administered at least about any of 1x, 2x, 3x, 4x, 5x, 6x, 7x (i.e., daily) a week. In some embodiments, the intervals between each administration are less than about any of 7 days, 6 days, 5 days, 4 days, 3 days, 2 days, and 1 day. In some embodiments, the nanoparticle composition is administered over a period of at least about any of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 18, 24, 30 and 36 months.

[0036] In some embodiments, there is provided a method of administering a composition comprising nanoparticles comprising a taxane and a carrier protein (such as albumin), wherein the taxane is administered over a period of at least one month, wherein the interval between each administration is no more than about a week, and wherein the dose of the taxane at each administration is about 0.25 mg/m^2 to about 25 mg/m^2. In some embodiments, there is provided a method of administering a composition comprising nanoparticles comprising paclitaxel and an albumin (such as Abraxane^{TM}) and a carrier protein (such as albumin), wherein the paclitaxel is administered over a period of at least one month, wherein the interval between each administration is no more than about a week, and wherein the dose of the taxane at each administration is about 0.25 mg/m^2 to about 25

mg/m^2. In some embodiments, the dose of the taxane (such as paclitaxel, for example Abraxane^{TM}) per administration is less than about any of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 18, 20, 22, and 25 mg/m^2. In some embodiments, the nanoparticle composition is administered at least about any of 1x, 2x, 3x, 4x, 5x, 6x, 7x (i.e., daily) a week. In some embodiments, the intervals between each administration are less than about any of 7 days, 6 days, 5 days, 4 days, 3 days, 2 days, and 1 day. In some embodiments, the nanoparticle composition is administered over a period of at least about any of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 18, 24, 30 and 36 months.

[0037] The methods of the invention generally comprise administration of a composition comprising nanoparticles comprising a taxane and a carrier protein. In some embodiments, the nanoparticle composition comprises nanoparticles comprising paclitaxel and an albumin. In some embodiments, the paclitaxel/albumin nanoparticles have an average diameter of no greater than about 200 nm. In some embodiments, the paclitaxel/albumin nanoparticle composition is substantially free (such as free) of surfactant (such as Cremophor). In some embodiments, the weight ratio of the albumin to paclitaxel in the composition is about 18:1 or less, such as about 9:1 or less. In some embodiments, the paclitaxel is coated with albumin. In some embodiments, the paclitaxel/albumin nanoparticles have an average diameter of no greater than about 200 nm and the paclitaxel/albumin composition is substantially free (such as free) of surfactant (such as Cremophor). In some embodiments, the paclitaxel/albumin nanoparticles have an average diameter of no greater than about 200 nm and the paclitaxel is coated with albumin. Other combinations of the above characteristics are also contemplated. In some embodiments, the nanoparticle composition is Abraxane^{TM}. Nanoparticle compositions comprising other taxanes (such as docetaxel and ortataxel) may also comprise one or more of the above characteristics.

[0038] These and other aspects and advantages of the present invention will become apparent from the subsequent detailed description and the appended claims. It is to be understood that one, some, or all of the properties of the various embodiments described herein may be combined to form other embodiments of the present invention.

BRIEF DESCRIPTION OF FIGURES

[0039] Figure 1A shows the effect of ABI-007 on rat aortic ring angiogenesis. Figure 1B shows the effect of ABI-007 on human endothelial cell proliferation. Figure 1C shows the effect of ABI-007 on endothelial cell tube formation.

[0040] Figure 2 shows the determination of an optimal biological dose of ABI-007 for metronomic dosing. Shown are the levels of viable circulating endothelial progenitors (CEPs) in peripheral blood of Balb/cJ mice in response to escalating doses of ABI-007. Untr'd, untreated control; S/A, saline/albumin vehicle control. Bars, mean ± SE. * Significantly ($p < 0.05$) different from the untreated control.

[0041] Figures 3A and 3B show the effects of ABI-007 and Taxol used in metronomic or MTD regimes on MDA-MB-231 (A) and PC3 (B) tumor growth tumor-bearing SCID mice. Figures 3C and 3D show the effects of ABI-007 and Taxol used in metronomic or MTD regimes on the body weight of MDA-MB-231 (C) and PC3 (D) tumor-bearing SCID mice.

[0042] Figures 4A and 4B show changes in the levels of viable circulating endothelial progenitors (CEPs) in peripheral blood of MDA-MB-231 (Fig. 4A) and PC3 (Fig. 4B) tumor-bearing SCID mice after treatment with A, saline/albumin; B, Cremophor EL control; C, metronomic Taxol 1.3 mg/kg; D, E, and F, metronomic ABI-007 3, 6, and 10 mg/kg, respectively; G, MTD Taxol; H, MTD ABI-007. Bars, mean ± SE. [a] Significantly ($p < 0.05$) different from saline/albumin vehicle control. [b] Significantly ($p < 0.05$) different from Cremophor EL vehicle control.

[0043] Figure 5A shows intratumoral microvessel density of MDA-MB-231 (■) and PC3 (□) xenografts treated with A, saline/albumin; B, Cremophor EL control; C, metronomic Taxol 1.3 mg/kg; D, E, and F, metronomic ABI-007 3, 6, and 10 mg/kg, respectively; G, MTD Taxol; H, MTD ABI-007. Bars, mean ± SE. Figure 5B and 5C show the correlation between intratumoral microvessel density and the number of viable CEPs in peripheral blood in MDA-MB-231 (Fig. 5B) and PC3 (Fig. 5C) tumor-bearing SCID mice.

[0044] Figure 6 shows the effects of ABI-007 or Taxol used in metronomic or MTD regimes on basic fibroblast growth factor (bFGF)-induced angiogenesis in matrigel plugs injected subcutaneously into the flanks of Balb/cJ mice. Treatments-A, saline/albumin; B, Cremophor EL control; C, metronomic Taxol 1.3 mg/kg; D, E, and F, metronomic ABI-007 3, 6, and 10 mg/kg, respectively; G, MTD Taxol; H, MTD ABI-007. Matrigel implanted without bFGF (-bFGF) served as negative control. Bars, mean ±SE.

[0045] Figure 7A and Figure 7B show the cytotoxic activity of nab-rapamycin in combination with Abraxane™ on vascular smooth muscle cells. Cytotoxicity was evaluated by staining with ethidium homodimer-1 (Fig. 7A) or by staining with calcein (Fig. 7B).

[0046] Figure 8 shows the cytotoxic activity of nab-rapamycin in combination with Abraxane™ in a HT29 human colon carcinoma xenograft model.

[0047] Figure 9 shows the cytotoxic activity of nab-17-AAG in combination with Abraxane™ in a H358 human lung carcinoma xenograft model.

DETAILED DESCRIPTION OF THE INVENTION

[0048] The present invention provides methods of combination therapy comprising a first therapy comprising administration of nanoparticles comprising a taxane and a carrier protein (such as albumin) in conjunction with a second therapy such as radiation, surgery, administration of at least one other chemotherapeutic agent, or combinations thereof. The invention also provides methods of metronomic therapy.

[0049] The present invention involves the discovery that Abraxane™, due to its superior anti-tumor activity and reduced toxicity and side effects, can be administered in combination with other therapeutic drugs and/or treatment modalities and can also be used in metronomic chemotherapy. Due to significantly improved safety profiles with compositions comprising drug/carrier protein nanoparticles (such as Abraxane™), we believe that combination chemotherapy with such nanoparticle compositions (such as Abraxane™) is more effective than combination chemotherapy with other drugs. In addition the use of nanoparticle composition (such as Abraxane™) in combination with radiation is also believed to be more effective than combination of other agents with radiation. Thus, the nanoparticle compositions (especially a paclitaxel/albumin nanoparticle composition, such as Abraxane™), when used in combination with other chemotherapeutic agents or when combined with other treatment modalities, should be very effective and overcome the deficiencies of surgery, radiation treatment, and chemotherapy in the treatment of proliferative disease (such as cancer).

[0050] The present invention in one its embodiments is the use of a first therapy comprising a taxane, such as Abraxane™, in combination with a second therapy such as another chemotherapeutic agent or agents, radiation, or the like for treating proliferative diseases such as cancer. The first therapy comprising a taxane and second therapy can be administered to a mammal having the proliferative sequentially, or they can be co-administered, and even administered simultaneously in the same pharmaceutical composition.

[0051] Further, a metronomic dosing regime using Abraxane™ has been found to be more effective than the traditional MTD dosing schedule of the same drug composition. Such metronomic dosing regime of Abraxane™ has also been found to be more effective than metronomic dosing of Taxol®.

[0052] The methods described herein are generally useful for treatment of diseases, particularly proliferative diseases. As used herein, "treatment" is an approach for obtaining beneficial or desired clinical results. For purposes of this invention, beneficial or desired clinical results include, but are not limited to, any one or more of: alleviation of one or more symptoms, diminishment of extent of disease, stabilized (i.e., not worsening) state of disease, preventing or delaying spread (e.g., metastasis) of disease, preventing or delaying occurrence or recurrence of disease, delay or slowing of disease progression, amelioration of the disease state, and remission (whether partial or total). Also encompassed by "treatment" is a reduction of pathological consequence of a proliferative disease. The methods of the invention contemplate any one or more of these aspects of treatment.

[0053] As used herein, a "proliferative disease" is defined as a tumor disease (including benign or cancerous) and/or any metastases, wherever the tumor or the metastasis are located, more especially a tumor selected from the group comprising one or more of (and in some embodiments selected from the group consisting of) breast cancer, genitourinary cancer, lung cancer, gastrointestinal cancer, epidermoid cancer, melanoma, ovarian cancer, pancreatic cancer, neuroblastoma, colorectal cancer, head and neck cancer. In a broader sense of the invention, a proliferative disease may furthermore be selected from hyperproliferative conditions such as hyperplasias, fibrosis (especially pulmonary, but also other types of fibrosis, such as renal fibrosis), angiogenesis, psoriasis, atherosclerosis and smooth muscle proliferation in the blood vessels, such as stenosis or restenosis following angioplasty. In some embodiments, the proliferative disease is cancer. In some embodiments, the proliferative disease is a non-cancerous disease. In some embodiments, the proliferative disease is a benign or malignant tumor. Where hereinbefore and subsequently a tumor, a tumor disease, a carcinoma or a cancer are mentioned, also metastasis in the original organ or tissue and/or in any other location are implied alternatively or in addition, whatever the location of the tumor and/or metastasis is.

[0054] The term "effective amount" used herein refers to an amount of a compound or composition sufficient to treat a specified disorder, condition or disease such as ameliorate, palliate, lessen, and/or delay one or more of its symptoms. In reference to cancers or other unwanted cell proliferation, an effective amount comprises an amount

sufficient to cause a tumor to shrink and/or to decrease the growth rate of the tumor (such as to suppress tumor growth) or to prevent or delay other unwanted cell proliferation. In some embodiments, an effective amount is an amount sufficient to delay development. In some embodiments, an effective amount is an amount sufficient to prevent or delay occurrence and/or recurrence. An effective amount can be administered in one or more administrations. In the case of cancer, the effective amount of the drug or composition may: (i) reduce the number of cancer cells; (ii) reduce tumor size; (iii) inhibit, retard, slow to some extent and preferably stop cancer cell infiltration into peripheral organs; (iv) inhibit (i.e., slow to some extent and preferably stop) tumor metastasis; (v) inhibit tumor growth; (vi) prevent or delay occurrence and/or recurrence of tumor; and/or (vii) relieve to some extent one or more of the symptoms associated with the cancer.

[0055] In some embodiments, there is provided a method of treating a primary tumor. In some embodiments, there is provided a method of treating metastatic cancer (that is, cancer that has metastasized from the primary tumor). In some embodiments, there is provided a method of treating cancer at advanced stage(s). In some embodiments, there is provided a method of treating breast cancer (which may be HER2 positive or HER2 negative), including, for example, advanced breast cancer, stage IV breast cancer, locally advanced breast cancer, and metastatic breast cancer. In some embodiments, there is provided a method of treating lung cancer, including, for example, non-small cell lung cancer (NSCLC, such as advanced NSCLC), small cell lung cancer (SCLC, such as advanced SCLC), and advanced solid tumor malignancy in the lung. In some embodiments, there is provided a method of treating any of ovarian cancer, head and neck cancer, gastric malignancies, melanoma (including metastatic melanoma), colorectal cancer, pancreatic cancer, and solid tumors (such as advanced solid tumors). In some embodiments, there is provided a method of reducing cell proliferation and/or cell migration. In some embodiments, there is provided a method of treating any of the following diseases: restenosis, stenosis, fibrosis, angiogenesis, psoriasis, atherosclerosis, and proliferation of smooth muscle cells. The present invention also provides methods of delaying development of any of the proliferative diseases described herein.

[0056] The term "individual" is a mammal, including humans. An individual includes, but is not limited to, human, bovine, horse, feline, canine, rodent, or primate. In some embodiments, the individual is human. The individual (such as human) may have advanced disease or lesser extent of disease, such as low tumor burden. In some embodiments, the individual is at an early stage of a proliferative disease (such as cancer).

In some embodiments, the individual is at an advanced stage of a proliferative disease (such as an advanced cancer). In some embodiments, the individual is HER2 positive. In some embodiments, the individual is HER2 negative.

[0057] The methods may be practiced in an adjuvant setting. "Adjuvant setting" refers to a clinical setting in which an individual has had a history of a proliferative disease, particularly cancer, and generally (but not necessarily) been responsive to therapy, which includes, but is not limited to, surgery (such as surgical resection), radiotherapy, and chemotherapy. However, because of their history of the proliferative disease (such as cancer), these individuals are considered at risk of development of the disease. Treatment or administration in the "adjuvant setting" refers to a subsequent mode of treatment. The degree of risk (i.e., when an individual in the adjuvant setting is considered as "high risk" or "low risk") depends upon several factors, most usually the extent of disease when first treated. The methods provided herein may also be practiced in a neoadjuvant setting, i.e., the method may be carried out before the primary/definitive therapy. In some embodiments, the individual has previously been treated. In some embodiments, the individual has not previously been treated. In some embodiments, the treatment is a first line therapy.

[0058] It is understood that aspect and embodiments of the invention described herein include "consisting" and/or "consisting essentially of" aspects and embodiments.

Combination therapy with chemotherapeutic agent

[0059] The present invention provides methods of treating a proliferative disease (such as cancer) in an individual, comprising administering to the individual: a) an effective amount of a composition comprising nanoparticles comprising a taxane and a carrier protein (such as albumin); and b) an effective amount of at least one other chemotherapeutic agent. In some embodiments, the taxane is any of (and in come embodiments consisting essentially of) paclitaxel, docetaxel, and ortataxel. In some embodiments, the nanoparticle composition comprises Abraxane™. In some embodiments, the chemotherapeutic agent is any of (and in some embodiments selected from the group consisting of) antimetabolite agents (including nucleoside analogs), platinum-based agents, alkylating agents, tyrosine kinase inhibitors, anthracycline antibiotics, vinca alkloids, proteasome inhibitors, macrolides, and topoisomerase inhibitors.

[0060] In some embodiments, the method comprises administering to the individual: a) an effective amount of a composition comprising nanoparticles comprising

paclitaxel and an albumin; and b) an effective amount of at least one other chemotherapeutic agent. In some embodiments, the paclitaxel/albumin nanoparticles have an average diameter of no greater than about 200 nm. In some embodiments, the paclitaxel/albumin nanoparticle composition is substantially free (such as free) of surfactant (such as Cremophor). In some embodiments, the weight ratio of the albumin to paclitaxel in the composition is about 18:1 or less, such as about 9:1 or less. In some embodiments, the paclitaxel is coated with albumin. In some embodiments, the paclitaxel/albumin nanoparticles have an average diameter of no greater than about 200 nm and the paclitaxel/albumin composition is substantially free (such as free) of surfactant (such as Cremophor). In some embodiments, the paclitaxel/albumin nanoparticles have an average diameter of no greater than about 200 nm and the paclitaxel is coated with albumin. In some embodiments, the nanoparticle composition is Abraxane™.

[0061] In some embodiments, the invention provides a method of treating a proliferative disease (such as cancer) in an individual comprising administering to the individual a) an effective amount of Abraxane™, and b) an effective amount of at least one other chemotherapeutic agent. Preferred drug combinations for sequential or co-administration or simultaneous administration with Abraxane™ are those which show enhanced antiproliferative activity when compared with the single components alone, especially combinations that that lead to regression of proliferative tissues and/or cure from proliferative diseases.

[0062] The chemotherapeutic agents described herein can be the agents themselves, pharmaceutically acceptable salts thereof, and pharmaceutically acceptable esters thereof, as well as steroisomers, enantiomers, racemic mixtures, and the like. The chemotherapeutic agent or agents as described can be administered as well as a pharmaceutical composition containing the agent(s), wherein the pharmaceutical composition comprises a pharmaceutically acceptable carrier vehicle, or the like.

[0063] The chemotherapeutic agent may be present in a nanoparticle composition. For example, in some embodiments, there is provided a method of treating a proliferative disease (such as cancer) in an individual, comprising administering to the individual: a) an effective amount of a composition comprising nanoparticles comprising a taxane and a carrier protein (such as albumin); and b) an effective amount of a composition comprising nanoparticles comprising at least one other chemotherapeutic agent and a carrier protein (such as albumin). In some embodiments, the method comprises administering to the individual: a) an effective amount of a composition comprising nanoparticles comprising

paclitaxel and an albumin (such as Abraxane™); and b) an effective amount of a composition comprising nanoparticles comprising at least one other chemotherapeutic agent and a carrier protein (such as albumin). In some embodiments, the chemotherapeutic agent is any of (and in some embodiments selected from the group consisting of) thiocolchicine or its derivatives (such as dimeric thiocolchicine, including for example *nab*-5404, *nab*-5800, and *nab*-5801), rapamycin or its derivatives, and geldanamycin or its derivatives (such as 17-allyl amino geldanamycin (17-AAG)). In some embodiments, the chemotherapeutic agent is rapamycin. In some embodiments, the chemotherapeutic agent is 17-AAG.

[0064] An exemplary and non-limiting list of chemotherapeutic agents contemplated is provided herein. Suitable chemotherapeutic agents include, for example, vinca alkaloids, agents that disrupt microtubule formation (such as colchicines and its derivatives), anti-angiogenic agents, therapeutic antibodies, EGFR targeting agents, tyrosine kinase targeting agent (such as tyrosine kinase inhibitors), transitional metal complexes, proteasome inhibitors, antimetabolites (such as nucleoside analogs), alkylating agents, platinum-based agents, anthracycline antibiotics, topoisomerase inhibitors, macrolides, therapeutic antibodies, retinoids (such as all-trans retinoic acids or a derivatives thereof); geldanamycin or a derivative thereof (such as 17-AAG), and other standard chemotherapeutic agents well recognized in the art.

[0065] In some embodiments, the chemotherapeutic agent is any of (and in some embodiments selected from the group consisting of) adriamycin, colchicine, cyclophosphamide, actinomycin, bleomycin, duanorubicin, doxorubicin, epirubicin, mitomycin, methotrexate, mitoxantrone, fluorouracil, carboplatin, carmustine (BCNU), methyl-CCNU, cisplatin, etoposide, interferons, camptothecin and derivatives thereof, phenesterine, taxanes and derivatives thereof (e.g., paclitaxel and derivatives thereof, taxotere and derivatives thereof, and the like), topetecan, vinblastine, vincristine, tamoxifen, piposulfan, nab-5404, nab-5800, nab-5801, Irinotecan, HKP, Ortataxel, gemcitabine, Herceptin®, vinorelbine, Doxil®, capecitabine, Alimta®, Avastin®, Velcade®, Tarceva®, Neulasta®, Lapatinib, Sorafenib, derivatives thereof, chemotherapeutic agents known in the art, and the like. In some embodiments, the chemotherapeutic agent is a composition comprising nanoparticles comprising a thiocolchicine derivative and a carrier protein (such as albumin).

[0066] In some embodiments, the chemotherapeutic agent is a antineoplastic agent including, but is not limited to, carboplatin, Navelbine® (vinorelbine), anthracycline

(Doxil®), lapatinib (GW57016), Herceptin®, gemcitabine (Gemzar®), capecitabine (Xeloda®), Alimta®, cisplatin, 5-fluorouracil, epirubicin, cyclophosphamide, Avastin®, Velcade®, etc.

[0067] In some embodiments, the chemotherapeutic agent is an antagonist of other factors that are involved in tumor growth, such as EGFR, ErbB2 (also known as Herb), ErbB3, ErbB4, or TNF. Sometimes, it may be beneficial to also administer one or more cytokines to the individual. In some embodiments, the therapeutic agent is a growth inhibitory agent. Suitable dosages for the growth inhibitory agent are those presently used and may be lowered due to the combined action (synergy) of the growth inhibitory agent and the taxane.

[0068] In some embodiments, the chemotherapeutic agent is a chemotherapeutic agent other than an anti-VEGF antibody, a HER2 antibody, interferon, and an HGFβ antagonist.

[0069] Reference to a chemotherapeutic agent herein applies to the chemotherapeutic agent or its derivatives and accordingly the invention contemplates and includes either of these embodiments (agent; agent or derivative(s)). "Derivatives" or "analogs" of a chemotherapeutic agent or other chemical moiety include, but are not limited to, compounds that are structurally similar to the chemotherapeutic agent or moiety or are in the same general chemical class as the chemotherapeutic agent or moiety. In some embodiments, the derivative or analog of the chemotherapeutic agent or moiety retains similar chemical and/or physical property (including, for example, functionality) of the chemotherapeutic agent or moiety.

[0070] In some embodiments, the invention provides a method of treating a proliferative disease (such as cancer) in an individual, comprising administering to the individual a) an effective amount of a composition comprising nanoparticles comprising a taxane and a carrier protein (such as albumin), and b) an effective amount of a tyrosine kinase inhibitor. In some embodiments, the invention provides a method of treating a proliferative disease (such as cancer) in an individual, comprising administering to the individual a) an effective amount of a composition comprising nanoparticles comprising paclitaxel and an albumin (such as Abraxane^{TM}), and b) an effective amount of a tyrosine kinase inhibitor. Suitable tyrosine kinase inhibitors include, for example, imatinib (Gleevec®), gefitinib (Iressa®), Tarceva, Sutent® (sunitinib malate), and Lapatinib. In some embodiments, the tyrosine kinase inhibitor is lapatinib. In some embodiments, the tyrosine kinase inhibitor is Tarceva. Tarceva is a small molecule human epidermal growth

factor type 1/epidermal growth factor receptor (HER1/EGFR) inhibitor which demonstrated, in a Phase III clinical trial, an increased survival in advanced non-small cell lung cancer (NSCLC) individuals. In some embodiments, the method is for treatment of breast cancer, including treatment of metastatic breast cancer and treatment of breast cancer in a neoadjuvant setting. In some embodiments, the method is for treatment of advanced solid tumor. In some embodiments, there is provided a method to inhibit the proliferation of EGFR expressing tumors in a mammal comprising administering to a mammal infected with such tumors Abraxane™ and gefitinib, wherein the gefitinib is administered by pulse-dosing.

[0071] In some embodiments, the invention provides a method of treating a proliferative disease (such as cancer) in an individual, comprising administering to the individual a) an effective amount of a composition comprising nanoparticles comprising a taxane and a carrier protein (such as albumin), and b) an effective amount of an antimetabolite agent (such as a nucleoside analog, including for example purine analogs and pyrimidine analogs). In some embodiments, the invention provides a method of treating a proliferative disease (such as cancer) in an individual, comprising administering to the individual a) an effective amount of a composition comprising nanoparticles comprising paclitaxel and an albumin (such as Abraxane™), and b) an effective amount of an antimetabolite agent. An "antimetabolic agent" is an agent which is structurally similar to a metabolite, but cannot be used by the body in a productive manner. Many antimetabolite agents interfere with production of nucleic acids, RNA and DNA. For example, the antimetabolite can be a nucleoside analog, which includes, but is not limited to, azacitidine, azathioprine, capecitabine (Xeloda®), cytarabine, cladribine, cytosine arabinoside (ara-C, cytosar), doxifluridine, fluorouracil (such as 5-fluorouracil), UFT, hydoxyurea, gemcitabine, mercaptopurine, methotrexate, thioguanine (such as 6-thioguanine). Other anti-metabolites include, for example, L-asparaginase (Elspa), decarbazine (DTIC), 2-deoxy-D-glucose, and procarbazine (matulane). In some embodiments, the nucleoside analog is any of (and in some embodiments selected from the group consisting of) gemcitabine, fluorouracil, and capecitabine. In some embodiments, the method is for treatment of metastatic breast cancer or locally advanced breast cancer. In some embodiments, the method is for first line treatment of metastatic breast cancer. In some embodiments, the method is for treatment of breast cancer in a neoadjuvant setting. In some embodiments, the method is for treatment of any of NSCLC, metastatic colorectal cancer, pancreatic cancer, or advanced solid tumor.

[0072] In some embodiments, the invention provides a method of treating a proliferative disease (such as cancer) in an individual, comprising administering to the individual a) an effective amount of a composition comprising nanoparticles comprising a taxane and a carrier protein (such as albumin), and b) an effective amount of an alkylating agent. In some embodiments, the invention provides a method of treating a proliferative disease (such as cancer) in an individual, comprising administering to the individual a) an effective amount of a composition comprising nanoparticles comprising paclitaxel and an albumin (such as Abraxane™), and b) an effective amount of an alkylating agent. Suitable alkylating agents include, but are not limited to, cyclophosphamide (Cytoxan), mechlorethamine, chlorambucil, melphalan, carmustine (BCNU), thiotepa, busulfan, alkyl sulphonates, ethylene imines, nitrogen mustard analogs, estramustine sodium phosphate, ifosfamide, nitrosoureas, lomustine, and streptozocin. In some embodiments, the alkylating agent is cyclophosphamide. In some embodiments, the cyclophosphamide is administered prior to the administration of the nanoparticle composition. In some embodiments, the method is for treatment of an early stage breast cancer. In some embodiments, the method is for treatment of a breast cancer in an adjuvant or a neoadjuvant setting.

[0073] In some embodiments, the invention provides a method of treating a proliferative disease (such as cancer) in an individual, comprising administering to the individual a) an effective amount of a composition comprising nanoparticles comprising a taxane and a carrier protein (such as albumin), and b) an effective amount of a platinum-based agent. In some embodiments, the invention provides a method of treating a proliferative disease (such as cancer) in an individual, comprising administering to the individual a) an effective amount of a composition comprising nanoparticles comprising paclitaxel and an albumin (such as Abraxane™), and b) an effective amount of a platinum-based agent. Suitable platinum-based agents include, but are not limited to, carboplatin, cisplatin, and oxaliplatin. In some embodiments, the platinum-based agent is carboplatin. In some embodiments, the method is for treatment of: breast cancer (HER2 positive or HER2 negative, including metastatic breast cancer and advanced breast cancer); lung cancer (including advanced NSCLC, first line NSCLC, SCLC, and advanced solid tumor malignancies in the lung); ovarian cancer; head and neck cancer; and melanoma (including metastatic melanoma).

[0074] In some embodiments, the invention provides a method of treating a proliferative disease (such as cancer) in an individual, comprising administering to the individual a) an effective amount of a composition comprising nanoparticles comprising a

taxane and a carrier protein (such as albumin), and b) an effective amount of an anthracycline antibiotic. In some embodiments, the invention provides a method of treating a proliferative disease (such as cancer) in an individual, comprising administering to the individual a) an effective amount of a composition comprising nanoparticles comprising paclitaxel and an albumin (such as Abraxane™) and a carrier protein (such as albumin), and b) an effective amount of an anthracycline antibiotic. Suitable anthracycline antibiotic include, but are not limited to, Doxil®, actinomycin, dactinomycin, daunorubicin (daunomycin), doxorubicin (adriamycin), epirubicin, idarubicin, mitoxantrone, valrubicin. In some embodiments, the anthracycline is any of (and in some embodiments selected from the group consisting of) Doxil®, epirubicin, and doxorubicin. In some embodiments, the method is for treatment of an early stage breast cancer. In some embodiments, the method is for treatment of a breast cancer in an adjuvant or a neoadjuvant setting.

[0075] In some embodiments, the invention provides a method of treating a proliferative disease (such as cancer) in an individual, comprising administering to the individual a) an effective amount of a composition comprising nanoparticles comprising a taxane and a carrier protein (such as albumin), and b) an effective amount of a vinca alkloid. In some embodiments, the invention provides a method of treating a proliferative disease (such as cancer) in an individual, comprising administering to the individual a) an effective amount of a composition comprising nanoparticles comprising palitaxel and an albumin (such as Abraxane™) and a carrier protein (such as albumin), and b) an effective amount of a vinca alkloid. Suitable vinca alkaloids include, for example, vinblastine, vincristine, vindesine, vinorelbine (Navelbine®), and VP-16. In some embodiments, the vinca alkaloid is vinorelbine (Navelbine®). In some embodiments, the method is for treatment of stage IV breast cancer and lung cancer.

[0076] In some embodiments, the invention provides a method of treating a proliferative disease (such as cancer) in an individual, comprising administering to the individual a) an effective amount of a composition comprising nanoparticles comprising a taxane and a carrier protein (such as albumin), and b) an effective amount of a macrolide. In some embodiments, the invention provides a method of treating a proliferative disease (such as cancer) in an individual, comprising administering to the individual a) an effective amount of a composition comprising nanoparticles comprising paclitaxel and an albumin (such as Abraxane™) and a carrier protein (such as albumin), and b) an effective amount of a macrolide. Suitable macrolides include, for example, rapamycin, carbomycin, and

erythromycin. In some embodiments, the macrolide is rapamycin or a derivative thereof. In some embodiments, the method is for treatment of a solid tumor.

[0077] In some embodiments, the invention provides a method of treating a proliferative disease (such as cancer) in an individual, comprising administering to the individual a) an effective amount of a composition comprising nanoparticles comprising a taxane and a carrier protein (such as albumin), and b) an effective amount of a topoisomerase inhibitor. In some embodiments, the invention provides a method of treating a proliferative disease (such as cancer) in an individual, comprising administering to the individual a) an effective amount of a composition comprising nanoparticles comprising paclitaxel and an albumin (such as Abraxane™) and a carrier protein (such as albumin), and b) an effective amount of a topoisomerase inhibitor. In some embodiments, the chemotherapeutic agent is a topoisomerase inhibitor, including, for example, inhibitor of topoisomerase I and topoisomerase II. Exemplary inhibitors of topoisomerase I include, but are not limited to, camptothecin, such as irinotecan and topotecan. Exemplary inhibitors of topoisomerase II include, but are not limited to, amsacrine, etoposide, etoposide phosphate, and teniposide.

[0078] In some embodiments, the invention provides a method of treating a proliferative disease (such as cancer) in an individual, comprising administering to the individual a) an effective amount of a composition comprising nanoparticles comprising a taxane and a carrier protein (such as albumin), and b) an effective amount of an antiangiogenic agent. In some embodiments, the invention provides a method of treating a proliferative disease (such as cancer) in an individual, comprising administering to the individual a) an effective amount of a composition comprising nanoparticles comprising paclitaxel and an albumin (such as Abraxane™) and a carrier protein (such as albumin), and b) an effective amount of an antiangiogenic agent. In some embodiments, the method is for treatment of metastatic breast cancer, breast cancer in an adjuvant setting or a neoadjuvant setting, lung cancer (such as first line advanced NSCLC and NSCLC), ovarian cancer, and melanoma (including metastatic melanoma).

[0079] Many anti-angiogenic agents have been identified and are known in the art, including those listed by Carmeliet and Jain (2000). The anti-angiogenic agent can be naturally occurring or non-naturally occurring. In some embodiments, the chemotherapeutic agent is a synthetic antiangiogenic peptide. For example, it has been previously reported that the antiangiogenic activity of small synthetic pro-apoptic peptides comprise two functional domains, one targeting the CD13 receptors (aminopeptidase N) on

tumor microvessels and the other disrupting the mitochondrial membrane following internalization. Nat. Med. 1999, 5(9):1032-8. A second generation dimeric peptide, CNGRC-GG-d(KLAKLAK)2, named HKP (Hunter Killer Peptide) was found to have improved antitumor activity. Accordingly, in some embodiments, the antiangiogenic peptide is HKP. In some embodiments, the antiangiogenic agent is other than an anti-VEGF antibody (such as Avastin®).

[0080] In some embodiments, the invention provides a method of treating a proliferative disease (such as cancer) in an individual, comprising administering to the individual a) an effective amount of a composition comprising nanoparticles comprising a taxane and a carrier protein (such as albumin), and b) an effective amount of a proteasome inhibitor, such as bortezomib (Velcade). In some embodiments, the invention provides a method of treating a proliferative disease (such as cancer) in an individual, comprising administering to the individual a) an effective amount of a composition comprising nanoparticles comprising paclitaxel and an albumin (such as Abraxane^{TM}) and a carrier protein (such as albumin), and b) an effective amount of a proteasome inhibitor such as bortezomib (Velcade).

[0081] In some embodiments, the invention provides a method of treating a proliferative disease (such as cancer) in an individual, comprising administering to the individual a) an effective amount of a composition comprising nanoparticles comprising a taxane and a carrier protein (such as albumin), and b) an effective amount of a therapeutic antibody. In some embodiments, the invention provides a method of treating a proliferative disease (such as cancer) in an individual, comprising administering to the individual a) an effective amount of a composition comprising nanoparticles comprising paclitaxel and an albumin (such as Abraxane^{TM}) and a carrier protein (such as albumin), and b) an effective amount of a therapeutic antibody. Suitable therapeutic antibodies include, but are not limited to, anti-VEGF antibody (such as Avastin® (bevacizumab)), anti-HER2 antibody (such as Herceptin® (trastuzumab)), Erbitux® (cetuximab), Campath (alemtuzumab), Myelotarg (gemtuzumab), Zevalin (ibritumomab tiuextan, Rituxan (rituximab), and Bexxar (tositumomab). In some embodiments, the chemotherapeutic agent is Erbitux® (cetuximab). In some embodiments, the chemotherapeutic agent is a therapeutic antibody other than an antibody against VEGF or HER2. In some embodiments, the method is for treatment of HER2 positive breast cancer, including treatment of advanced breast cancer, treatment of metastatic cancer, treatment of breast cancer in an adjuvant setting, and treatment of cancer in a neoadjuvant setting. In some embodiments, the method is for

treatment of any of metastatic breast cancer, breast cancer in an adjuvant setting or a neoadjuvant setting, lung cancer (such as first line advanced NSCLC and NSCLC), ovarian cancer, head and neck cancer, and melanoma (including metastatic melanoma). For example, in some embodiments, there is provided a method for treatment of HER2 positive metastatic breast cancer in an individual, comprising administering to the individual 125 mg/m^2 paclitaxel/albumin nanoparticle composition (such as Abraxane^{TM}) weekly for three weeks with the fourth week off, concurrent with the administration of Herceptin®.

[0082] In some embodiments, two or more chemotherapeutic agents are administered in addition to the taxane in the nanoparticle composition. These two or more chemotherapeutic agents may (but not necessarily) belong to different classes of chemotherapeutic agents. Examples of these combinations are provided herein. Other combinations are also contemplated.

[0083] In some embodiments, there is provided a method of treating a proliferative disease (such as cancer) in an individual, comprising administering to the individual a) an effective amount of a composition comprising nanoparticles comprising a taxane and a carrier protein (such as albumin), b) an effective amount of an antimetabolite (such as a nucleoside analog, for example, gemcitabine), and c) an anthracycline antibiotic (such as epirubicin). In some embodiments, there is provided a method of treating a proliferative disease (such as cancer) in an individual, comprising administering to the individual a) an effective amount of a composition comprising nanoparticles comprising paclitaxel and an albumin (such as Abraxane^{TM}), b) an effective amount of an antimetabolite (such as a nucleoside analog, for example, gemcitabine), and c) an effective amount of an anthracycline antibiotic (such as epirubicin). In some embodiments, the method is for treatment of breast cancer in a neoadjuvant setting. For example, in some embodiments, there is provided a method of treating locally advanced/inflammatory cancer in an individual comprising administering to the individual 220 mg/m^2 paclitaxel/albumin nanoparticle composition (such as Abraxane^{TM}) every two weeks; 2000 mg/m^2 gemcitabine, every two weeks; and 50 mg/m^2 epirubicin, every two weeks. In some embodiments, there is provided a method of treating breast cancer in an individual in an adjuvant setting, comprising administering to the individual 175 mg/m^2 paclitaxel/albumin nanoparticle composition (such as Abraxane^{TM}) every two weeks, 2000 mg/m^2 gemcitabine, every two weeks, and 50 mg/m^2 epirubicin, every two weeks.

[0084] In some embodiments, there is provided a method of treating a proliferative disease (such as cancer) in an individual, comprising administering to the individual a) an

effective amount of a composition comprising nanoparticles comprising a taxane and a carrier protein (such as albumin), b) an effective amount of a platinum-based agent (such as carboplatin), and c) a therapeutic antibody (such as ant-HER2 antibody (such as Herceptin®) and anti-VEGF antibody (such as Avastin®)). In some embodiments, there is provided a method of treating a proliferative disease (such as cancer) in an individual, comprising administering to the individual a) an effective amount of a composition comprising nanoparticles comprising paclitaxel and an albumin (such as Abraxane™), b) an effective amount of a platinum-based agent (such as carboplatin), and c) a therapeutic antibody (such as ant-HER2 antibody (such as Herceptin®) and anti-VEGF antibody (such as Avastin®)). In some embodiments, the method is for treatment of any of advanced breast cancer, metastatic breast cancer, breast cancer in an adjuvant setting, and lung cancer (including NSCLC and advanced NSCLC). In some embodiments, there is provided a method of treating metastatic cancer in an individual, comprising administering to the individual 75 mg/m^2 paclitaxel/albumin nanoparticle composition (such as Abraxane™) and carboplatin, AUC=2, wherein the administration is carried out weekly for three weeks with the fourth week off. In some embodiments, the method further comprises weekly administering about 2-4 mg/kg of Herception®.

[0085] In some embodiments, there is provided a method of treating a proliferative disease (such as cancer) in an individual, comprising administering to the individual a) an effective amount of a composition comprising nanoparticles comprising a taxane and a carrier protein (such as albumin), b) an effective amount of a platinum-based agent (such as carboplatin), and c) a vinca alkaloid (such as Navelbine®). In some embodiments, there is provided a method of treating a proliferative disease (such as cancer) in an individual, comprising administering to the individual a) an effective amount of a composition comprising nanoparticles comprising paclitaxel and an albumin (such as Abraxane™), b) an effective amount of a platinum-based agent (such as carboplatin), and c) a vinca alkaloid (such as Navelbine®). In some embodiments, the method is for treatment of lung cancer.

[0086] In some embodiments, the invention provides a method of treating a proliferative disease (such as cancer) in an individual, comprising administering to the individual a) an effective amount of a composition comprising nanoparticles comprising a taxane and a carrier protein (such as albumin), b) an effective amount of an alkylating agent (such as cyclophosphamide) and c) an anthracycline antibiotic (such as adriamycin). In some embodiments, the invention provides a method of treating a proliferative disease (such as cancer) in an individual, comprising administering to the individual a) an effective

amount of a composition comprising nanoparticles comprising paclitaxel and an albumin, b) an effective amount of an alkylating agent (such as cyclophosphamide) and c) an anthracycline antibiotic (such as adriamycin). In some embodiments, the method is for treatment of an early stage breast cancer. In some embodiments, the method is for treatment of a breast cancer in an adjuvant or a neoadjuvant setting. For example, in some embodiments, there is provided a method of treating an early stage breast cancer in an individual, comprising administering 260 mg/m^2 paclitaxel/albumin nanoparticle composition (such as Abraxane^{TM}), 60 mg/m^2 adriamycin, and 600 mg/m^2 cyclophosphamide, wherein the administration is carried out once every two weeks.

[0087] Other embodiments are provided in Table 1. For example, in some embodiments, there is provided a method of treating advanced breast cancer in an individual, comprising administering to the individual a) an effective amount of a composition comprising nanoparticles comprising a paclitaxel and an albumin (such as Abraxane^{TM}), b) an effective amount of carboplatin. In some embodiments, the method further comprises administering an effective amount of Herceptin$^{®}$ to the individual. In some embodiments, there is provided a method of treating metastatic breast cancer in an individual, comprising administering to the individual a) an effective amount of a composition comprising nanoparticles comprising paclitaxel and an albumin (such as Abraxane^{TM}), b) an effective amount of gemcitabine. In some embodiments, there is provided a method of treating advanced non-small cell lung cancer in an individual, comprising administering to the individual a) an effective amount of a composition comprising nanoparticles comprising paclitaxel and an albumin (such as Abraxane^{TM}), b) an effective amount of carboplatin.

[0088] In some embodiments, there is provided a composition comprising nanoparticles comprising a taxane (such as paclitaxel, docetaxel, or ortataxel) and a carrier protein (such as albumin) and at least one other chemotherapeutic agent. The compositions described herein may comprise effective amounts of the taxane and the chemotherapeutic agent for the treatment of a proliferative disease (such as cancer). In some embodiments, the chemotherapeutic agent and the taxane are present in the composition at a predetermined ratio, such as the weight ratios described herein. In some embodiments, the invention provides a synergistic composition of an effective amount of a composition comprising nanoparticles comprising a taxane (such as paclitaxel, docetaxel, or ortataxel) and an effective amount of at least one other chemotherapeutic agent.

[0089] In some embodiments, the invention provides pharmaceutical compositions comprising nanoparticles comprising a taxane and a carrier protein (such as albumin) for use in the treatment of a proliferative disease (such as cancer), wherein said use comprises simultaneous and/or sequential administration of at least one other chemotherapeutic agent. In some embodiments, the invention provides a pharmaceutical composition comprising a chemotherapeutic agent for use in the treatment of a proliferative disease (such as cancer), wherein said use comprises simultaneous and/or sequential administration of a composition comprising nanoparticles comprising a taxane and a carrier protein (such as albumin). In some embodiments, the invention provides taxane-containing nanoparticle compositions and compositions comprising one other chemotherapeutic agent for simultaneous, and/or sequential use for treatment of a proliferative disease (such as cancer).

Modes of administration

[0090] The composition comprising nanoparticles comprising taxane (also referred to as "nanoparticle composition") and the chemotherapeutic agent can be administered simultaneously (i.e., simultaneous administration) and/or sequentially (i.e., sequential administration).

[0091] In some embodiments, the nanoparticle composition and the chemotherapeutic agent (including the specific chemotherapeutic agents described herein) are administered simultaneously. The term "simultaneous administration," as used herein, means that the nanoparticle composition and the chemotherapeutic agent are administered with a time separation of no more than about 15 minute(s), such as no more than about any of 10, 5, or 1 minutes. When the drugs are administered simultaneously, the drug in the nanoparticles and the chemotherapeutic agent may be contained in the same composition (e.g., a composition comprising both the nanoparticles and the chemotherapeutic agent) or in separate compositions (e.g., the nanoparticles are contained in one composition and the chemotherapeutic agent is contained in another composition). For example, the taxane and the chemotherapeutic agent may be present in a single composition containing at least two different nanoparticles, wherein some of the nanoparticles in the composition comprise the taxane and a carrier protein, and some of the other nanoparticles in the composition comprise the chemotherapeutic agent and a carrier protein. The invention contemplates and encompasses such compositions. In some embodiments, only the taxane is contained in nanoparticles. In some embodiments, simultaneous administration of the drug in the

nanoparticle composition and the chemotherapeutic agent can be combined with supplemental doses of the taxane and/or the chemotherapeutic agent.

[0092] In some embodiments, the nanoparticle composition and the chemotherapeutic agent are administered sequentially. The term "sequential administration" as used herein means that the drug in the nanoparticle composition and the chemotherapeutic agent are administered with a time separation of more than about 15 minutes, such as more than about any of 20, 30, 40, 50, 60 or more minutes. Either the nanoparticle composition or the chemotherapeutic agent may be administered first. The nanoparticle composition and the chemotherapeutic agent are contained in separate compositions, which may be contained in the same or different packages.

[0093] In some embodiments, the administration of the nanoparticle composition and the chemotherapeutic agent are concurrent, i.e., the administration period of the nanoparticle composition and that of the chemotherapeutic agent overlap with each other. In some embodiments, the administration of the nanoparticle composition and the chemotherapeutic agent are non-concurrent. For example, in some embodiments, the administration of the nanoparticle composition is terminated before the chemotherapeutic agent is administered. In some embodiments, the administration of the chemotherapeutic agent is terminated before the nanoparticle composition is administered. The time period between these two non-concurrent administrations can range from about two to eight weeks, such as about four weeks.

[0094] The dosing frequency of the drug-containing nanoparticle composition and the chemotherapeutic agent may be adjusted over the course of the treatment, based on the judgment of the administering physician. When administered separately, the drug-containing nanoparticle composition and the chemotherapeutic agent can be administered at different dosing frequency or intervals. For example, the drug-containing nanoparticle composition can be administered weekly, while a chemotherapeutic agent can be administered more or less frequently. In some embodiments, sustained continuous release formulation of the drug-containing nanoparticle and/or chemotherapeutic agent may be used. Various formulations and devices for achieving sustained release are known in the art.

[0095] The nanoparticle composition and the chemotherapeutic agent can be administered using the same route of administration or different routes of administration. In some embodiments (for both simultaneous and sequential administrations), the taxane in the nanoparticle composition and the chemotherapeutic agent are administered at a

predetermined ratio. For example, in some embodiments, the ratio by weight of the taxane in the nanoparticle composition and the chemotherapeutic agent is about 1 to 1. In some embodiments, the weight ratio may be between about 0.001 to about 1 and about 1000 to about 1, or between about 0.01 to about 1 and 100 to about 1. In some embodiments, the ratio by weight of the taxane in the nanoparticle composition and the chemotherapeutic agent is less than about any of 100:1, 50:1, 30:1, 10:1, 9:1, 8:1, 7:1, 6:1, 5:1, 4:1, 3:1, 2:1, and 1:1 In some embodiments, the ratio by weight of the taxane in the nanoparticle composition and the chemotherapeutic agent is more than about any of 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, 30:1, 50:1, 100:1. Other ratios are contemplated.

[0096] The doses required for the taxane and/or the chemotherapeutic agent may (but not necessarily) be lower than what is normally required when each agent is administered alone. Thus, in some embodiments, a subtherapeutic amount of the drug in the nanoparticle composition and/or the chemotherapeutic agent are administered. "Subtherapeutic amount" or "subtherapeutic level" refer to an amount that is less than the therapeutic amount, that is, less than the amount normally used when the drug in the nanoparticle composition and/or the chemotherapeutic agent are administered alone. The reduction may be reflected in terms of the amount administered at a given administration and/or the amount administered over a given period of time (reduced frequency).

[0097] In some embodiments, enough chemotherapeutic agent is administered so as to allow reduction of the normal dose of the drug in the nanoparticle composition required to effect the same degree of treatment by at least about any of 5%, 10%, 20%, 30%, 50%, 60%, 70%, 80%, 90%, or more. In some embodiments, enough drug in the nanoparticle composition is administered so as to allow reduction of the normal dose of the chemotherapeutic agent required to effect the same degree of treatment by at least about any of 5%, 10%, 20%, 30%, 50%, 60%, 70%, 80%, 90%, or more.

[0098] In some embodiments, the dose of both the taxane in the nanoparticle composition and the chemotherapeutic agent are reduced as compared to the corresponding normal dose of each when administered alone. In some embodiments, both the taxane in the nanoparticle composition and the chemotherapeutic agent are administered at a subtherapeutic, i.e., reduced, level. In some embodiments, the dose of the nanoparticle composition and/or the chemotherapeutic agent is substantially less than the established maximum toxic dose (MTD). For example, the dose of the nanoparticle composition and/or the chemotherapeutic agent is less than about 50%, 40%, 30%, 20%, or 10% of the MTD.

[0099] A combination of the administration configurations described herein can be used. The combination therapy methods described herein may be performed alone or in conjunction with another therapy, such as surgery, radiation, chemotherapy, immunotherapy, gene therapy, and the like. Additionally, a person having a greater risk of developing the proliferative disease may receive treatments to inhibit or and/or delay the development of the disease.

[0100] As will be understood by those of ordinary skill in the art, the appropriate doses of chemotherapeutic agents will be approximately those already employed in clinical therapies wherein the chemotherapeutic agent are administered alone or in combination with other chemotherapeutic agents. Variation in dosage will likely occur depending on the condition being treated. As described above, in some embodiments, the chemotherapeutic agents may be administered at a reduced level.

[0101] The nanoparticle compositions described herein can be administered to an individual (such as human) via various routes, such as parenterally, including intravenous, intra-arterial, intraperitoneal, intrapulmonary, oral, inhalation, intravesicular, intramuscular, intra-tracheal, subcutaneous, intraocular, intrathecal, or transdermal. For example, the nanoparticle composition can be administered by inhalation to treat conditions of the respiratory tract. The composition can be used to treat respiratory conditions such as pulmonary fibrosis, broncheolitis obliterans, lung cancer, bronchoalveolar carcinoma, and the like. In some embodiments, the nanoparticle composition is administrated intravenously. In some embodiments, the nanoparticle composition is administered orally.

[0102] The dosing frequency of the administration of the nanoparticle composition depends on the nature of the combination therapy and the particular disease being treated. An exemplary dosing frequency include, but is not limited to, weekly without break; weekly, three out of four weeks; once every three weeks; once every two weeks; weekly, two out of three weeks. See also Table 1.

[0103] The dose of the taxane in the nanoparticle composition will vary with the nature of the combination therapy and the particular disease being treated. The dose should be sufficient to effect a desirable response, such as a therapeutic or prophylactic response against a particular disease. An exemplary dose of the taxane (in some embodiments paclitaxel) in the nanoparticle composition include, but is not limited to, about any of 50 mg/m^2, 60 mg/m^2, 75 mg/m^2, 80 mg/m^2, 90 mg/m^2, 100 mg/m^2, 120 mg/m^2, 160 mg/m^2, 175 mg/m^2, 200 mg/m^2, 210 mg/m^2, 220 mg/m^2, 260 mg/m^2, and 300 mg/m^2. For example, the dosage of paclitaxel in a nanoparticle composition can be in the range of 100-400

mg/m^2 when given on a 3 week schedule, or 50-250 mg/m^2 when given on a weekly schedule. See also Table 1.

[0104] Other exemplary dosing schedules for the administration of the nanoparticle composition (such as paclitaxel/albumin nanoparticle composition, for example Abraxane™) include, but are not limited to, 100 mg/m^2, weekly, without break; 75 mg/m^2 weekly, 3 out of four weeks; 100 mg/m^2, weekly, 3 out of 4 weeks; 125 mg/m^2, weekly, 3 out of 4 weeks; 125 mg/m^2, weekly, 2 out of 3 weeks; 130 mg/m^2, weekly, without break; 175 mg/m^2, once every 2 weeks; 260 mg/m^2, once every 2 weeks; 260 mg/m^2, once every 3 weeks; 180-300 mg/m^2, every three weeks; 60-175 mg/m^2, weekly, without break. In addition, the taxane (alone or in combination therapy) can be administered by following a metronomic dosing regime described herein.

[0105] Exemplary dosing regimes for the combination therapy of nanoparticle composition (such as paclitaxel/albumin nanoparticle composition, for example Abraxane™) and other agents include, but are not limited to, 125 mg/m^2 weekly, two out of three weeks, plus 825 mg/m^2 Xeloda®, daily; 260 mg/m^2 once every two weeks, plus 60 mg/m^2 adriamycin and 600 mg/m^2 cyclophosphamide, once every two weeks; 220-340 mg/m^2 once every three weeks, plus carboplatin, AUC=6, once every three weeks; 100-150 mg/m^2 weekly, plus carboplatin, AUC=6, once every three weeks; 175 mg/m2 once every two weeks, plus 2000 mg/m^2 gemcitabine and 50 mg/m^2 epirubicin, once every two weeks; and 75 mg/m^2 weekly, three out of four weeks, plus carboplatin, AUC=2, weekly, three out of four weeks.

[0106] In some embodiments, the nanoparticle composition of the taxane and the chemotherapeutic agent is administered according to any of the dosing regimes described in Table 1.

[0107] In some embodiments, there is provided a method of treating breast cancer in an individual comprising administering to the individual: a) an effective amount of a composition comprising nanoparticles comprising a taxane (such as paclitaxel) and an albumin, and b) an effective amount of at least one other chemotherapeutic agent as provided in Rows 1 to 35 in Table 1. In some embodiments, the administration of the nanoparticle composition and the chemotherapeutic agent may be any of the dosing regimes as indicated in Rows 1 to 35 in Table 1. In some embodiments, there is provided a method of treating metastatic breast cancer in an individual comprising administering to the individual: a) an effective amount of a composition comprising nanoparticles comprising a taxane (such as paclitaxel) and an albumin, and b) an effective amount of at least one other

chemotherapeutic agent as provided in Rows 2, 4-8, and 10-15 in Table 1. In some embodiments, the administration of the nanoparticle composition and the chemotherapeutic agent may be any of the dosing regimes as indicated in Rows 2, 4-8, and 10-15 in Table 1.

[0108] In some embodiments, there is provided a method of treating advanced breast cancer in an individual comprising administering to the individual: a) an effective amount of a composition comprising nanoparticles comprising a taxane (such as paclitaxel) and an albumin, and b) an effective amount of at least one other chemotherapeutic agent as provided in Rows 1 and 16 in Table 1. In some embodiments, the administration of the nanoparticle composition and the chemotherapeutic agent may be any of the dosing regimes as indicated in Rows 1 and 16 in Table 1. In some embodiments, there is provided a method of treating stage IV breast cancer in an individual comprising administering to the individual: a) an effective amount of a composition comprising nanoparticles comprising a taxane (such as paclitaxel) and an albumin, and b) an effective amount of at least one other chemotherapeutic agent as provided in Row 3 in Table 1. In some embodiments, the administration of the nanoparticle composition and the chemotherapeutic agent may be the dosing regime as indicated in Row 3 in Table 1.

[0109] In some embodiments, there is provided a method of treating breast cancer in an individual in an adjuvant setting comprising administering to the individual: a) an effective amount of a composition comprising nanoparticles comprising a taxane (such as paclitaxel) and an albumin, and b) an effective amount of at least one other chemotherapeutic agent as provided in Rows 18 to 24 in Table 1. In some embodiments, the administration of the nanoparticle composition and the chemotherapeutic agent may be any of the dosing regimes as indicated in Rows 18 to 24 in Table 1.

[0110] In some embodiments, there is provided a method of treating breast cancer in an individual in a neoadjuvant setting comprising administering to the individual: a) an effective amount of a composition comprising nanoparticles comprising a taxane (such as paclitaxel) and an albumin, and b) an effective amount of at least one other chemotherapeutic agent as provided in Rows 25 to 35 in Table 1. In some embodiments, the administration of the nanoparticle composition and the chemotherapeutic agent may be any of the dosing regimes as indicated in Rows 25 to 35 in Table 1.

[0111] In some embodiments, there is provided a method of treating lung cancer in an individual comprising administering to the individual: a) an effective amount of a composition comprising nanoparticles comprising a taxane (such as paclitaxel) and an albumin, and b) an effective amount of at least one other chemotherapeutic agent as

provided in Rows 36 to 48 in Table 1. In some embodiments, the administration of the nanoparticle composition and the chemotherapeutic agent may be any of the dosing regimes as indicated in Rows 36 to 48 in Table 1.

[0112] In some embodiments, there is provided a method of treating NSCLC (including advanced NSCLC and first line NSCLC) in an individual comprising administering to the individual: a) an effective amount of a composition comprising nanoparticles comprising a taxane (such as paclitaxel) and an albumin, and b) an effective amount of at least one other chemotherapeutic agent as provided in Rows 36-40 and 42-43 in Table 1. In some embodiments, the administration of the nanoparticle composition and the chemotherapeutic agent may be any of the dosing regimes as indicated in Rows 36-40 and 42-43 in Table 1. In some embodiments, there is provided a method of treating advanced solid tumor malignancy in the lung in an individual comprising administering to the individual: a) an effective amount of a composition comprising nanoparticles comprising a taxane (such as paclitaxel) and an albumin, and b) an effective amount of at least one other chemotherapeutic agent as provided in Row 41 in Table 1. In some embodiments, the administration of the nanoparticle composition and the chemotherapeutic agent may be the dosing regimes as indicated in Row 41 in Table 1. In some embodiments, there is provided a method of treating SCLC in an individual comprising administering to the individual: a) an effective amount of a composition comprising nanoparticles comprising a taxane (such as paclitaxel) and an albumin, and b) an effective amount of at least one other chemotherapeutic agent as provided in Row 48 in Table 1. In some embodiments, the administration of the nanoparticle composition and the chemotherapeutic agent may be the dosing regimes as indicated in Row 48 in Table 1.

[0113] In some embodiments, there is provided a method of treating ovarian cancer in an individual comprising administering to the individual: a) an effective amount of a composition comprising nanoparticles comprising a taxane (such as paclitaxel) and an albumin, and b) an effective amount of at least one other chemotherapeutic agent as provided in Rows 49 to 52 in Table 1. In some embodiments, the administration of the nanoparticle composition and the chemotherapeutic agent may be any of the dosing regimes as indicated in Rows 49 to 52 in Table 1.

[0114] In some embodiments, there is provided a method of treating head and neck cancer in an individual comprising administering to the individual: a) an effective amount of a composition comprising nanoparticles comprising a taxane (such as paclitaxel) and an albumin, and b) an effective amount of at least one other chemotherapeutic agent as

provided in Rows 53 to 55 in Table 1. In some embodiments, the administration of the nanoparticle composition and the chemotherapeutic agent may be any of the dosing regimes as indicated in Rows 53 to 55 in Table 1.

[0115] In some embodiments, there is provided a method of treating solid tumor (including advanced solid tumor) in an individual comprising administering to the individual: a) an effective amount of a composition comprising nanoparticles comprising a taxane (such as paclitaxel) and an albumin, and b) an effective amount of at least one other chemotherapeutic agent as provided in Rows 56 to 59 in Table 1. In some embodiments, the administration of the nanoparticle composition and the chemotherapeutic agent may be any of the dosing regimes as indicated in Rows 56 to 59 in Table 1.

[0116] In some embodiments, there is provided a method of treating melanoma (including metastatic melanoma) in an individual comprising administering to the individual: a) an effective amount of a composition comprising nanoparticles comprising a taxane (such as paclitaxel) and an albumin, and b) an effective amount of at least one other chemotherapeutic agent as provided in Rows 60-63 in Table 1. In some embodiments, the administration of the nanoparticle composition and the chemotherapeutic agent may be any of the dosing regimes as indicated in Rows 60 to 63 in Table 1.

[0117] In some embodiments, there is provided a method of treating metastatic colorectal cancer in an individual comprising administering to the individual: a) an effective amount of a composition comprising nanoparticles comprising a taxane (such as paclitaxel) and an albumin, and b) an effective amount of at least one other chemotherapeutic agent as provided in Row 64 in Table 1. In some embodiments, the administration of the nanoparticle composition and the chemotherapeutic agent may be the dosing regime as indicated in Row 64 in Table 1.

[0118] In some embodiments, there is provided a method of treating pancreatic cancer in an individual comprising administering to the individual: a) an effective amount of a composition comprising nanoparticles comprising a taxane (such as paclitaxel) and an albumin, and b) an effective amount of at least one other chemotherapeutic agent as provided in Rows 65 to 66 in Table 1. In some embodiments, the administration of the nanoparticle composition and the chemotherapeutic agent may be any of the dosing regimes as indicated in Rows 65 to 66 in Table 1.

TABLE 1

Row No.	Combination	Regime/Dosage	Study therapy type	Protocol title

Row No.	Combination	Regime/Dosage	Study therapy type	Protocol title
1.	ABX + Carboplatin + Herceptin®	ABX: 100 mg/m^2 D1, 8, 15 q4wk x 6 Carbo: AUC = 2 D1, 8, 15 q4wk x 6 Herceptin®: 4 mg/kg on wk 1, 2 mg/kg all subsequent weeks	Advanced HER2+ Breast Cancer	A phase II study of weekly dose-dense nanoparticle paclitaxel (ABI-007) carboplatin™, with Herceptin® as first or second-line therapy of advanced HER2+ breast cancer
2.	ABX alone (+Herceptin®)	ABX: 125 mg/m^2 qwk x 3/4	Metastatic Breast Cancer	Phase II trial of weekly Abraxane™ monotherapy for 1st-line MBC (plus Herceptin® in HER2+ pts)
3.	ABX + Navelbine® (±G-CSF)	L1: ABX: 80 mg/m Nav: 15 mg/m^2 L2: ABX: 90 mg/m^2 Nav: 20 mg/m^2 L3: ABX: 100 mg/m^2 Nav: 22.5 mg/m^2 L4: ABX: 110 mg/m^2 Nav: 25 mg/m^2 L5: ABX: 125 mg/m^2 Nav: 25 mg/m^2 qwk all levels	Stage IV Breast Cancer	Phase I-II study weekly ABX + Navelbine®, with or without G-CSF, in stage IV breast cancer
4.	ABX + Xeloda®	ABX: 125 mg/m^2 qwk x 2/3 Xeloda®: 825 mg/m^2 D1-14 q3wk	Metastatic Breast Cancer	Phase II 1st-line ABX + Xeloda® MBC trial
5.	ABX + Anthracycline		Metastatic Breast Cancer	Phase I/II trial ABX plus Doxil® for MBC plus limited PK
6.	ABX + Gemcitabine	ABX: 125 mg/m^2 Gem: 1000 mg/m2 qwk x 2/3	Metastatic Breast Cancer	Randomized Phase II Trial of Weekly nab (nanoparticle albumin bound)-Paclitaxel (nab-paclitaxel) in Combination with Gemcitabine in Patients with HER2 Negative Metastatic Breast Cancer
7.	ABX + Lapatinib		Metastatic Breast Cancer	Phase I/II Abraxane™ + GW572016
8.	ABX + Lapatinib	ABX: 100 mg/m^2 qwk x 3/4 Lapatinib: starting at 1000 mg/d x 2 days	Metastatic Breast Cancer	Phase I dose escalation study of a 2 day oral lapatinib chemosensitization

Row No.	Combination	Regime/Dosage	Study therapy type	Protocol title
				pulse given prior to weekly intravenous Abraxane™ in patients with advanced solid tumors
9.	ABX +FEC (+Herceptin®)	ABX: 220 mg/m^2 q2wk x 6 followed by FEC: 4 cycles (+Herceptin® for HER2+ pts)	Breast Cancer	Phase II preoperative trial of Abraxane™ followed by FEC (+Herceptin® as appropriate) in breast cancer
10.	ABX + Carboplatin + Avastin®	ABX: 100 mg/m^2 qwk D1, 8, 15 Carbo: AUC = 2 qwk D1, 8, 15 Avastin®: 10 mg/m^2 q2wk	Metastatic Breast Cancer (HER2-, ER-, PR-)	Phase II safety and tolerability study of Abraxane™, Avastin® and carboplatin in triple negative metastatic breast cancer patients
11.	ABX + Avastin®	ABX: 130 mg/m^2 qwk + Avastin® vs ABX: 260 mg/m^2 q2wk + Avastin® vs ABX: 260 mg/m^2 q3wk + Avastin®	Metastatic Breast Cancer	Three arm phase II trial in 1st line HER2-negative MBC patients
12.	ABX + Avastin®	ABX: 125 mg/m^2 qwk x 3/4 + Avastin®	Metastatic Breast Cancer	Single arm study of Abraxane™ and Avastin® in 1st line MBS
13.	ABX + Avastin®	ABX + Avastin® qwk vs Taxol® + Avastin® qwk	Metastatic Breast Cancer	Randomized Phase III trial in 1st line and 2nd line MBC with biological correlates analysis
14.	ABX + Xeloda® + Lapatinib		Metastatic Breast Cancer	Phase II Abraxane™ in combination with Xeloda® and Lapatinib for metastatic breast cancer
15.	ABX + Gemcitabine	ABX: 3000 mg/m^2 D1 q3wk Gem: 1250 mg/m^2 D1, 8 q3wk	Metastatic Breast Cancer	Single arm Phase II study of Abraxane™ and gemcitabine for 1st line MBC
16.	ABX + RAD001		Advanced Breast Cancer	Phase I/II study of Abraxane™ in combination with RAD001 in patients with advanced breast cancer

Row No.	Combination	Regime/Dosage	Study therapy type	Protocol title
17.	ABX + Sutent®		Breast Cancer	Phase I study of Abraxane™ in combination with Sutent®
18.	ABX + AC + G-CSF (+ Herceptin®)	AC + G-CSF q2wk x 4 followed by ABX: 260 mg/m^2 q2wk x 4 (+ Herceptin® for HER2+ pts)	Breast Cancer-Adjuvant	Abraxane™ in dose-dense adjuvant chemotherapy for early stage breast cancer
19.	ABX + AC + G-CSF (+ Herceptin®)	Dose dense AC + G-CSF followed by ABX (+ Herceptin® for HER2+ pts) qwk	Breast Cancer-Adjuvant	Phase II pilot adjuvant trial of Abraxane™ in breast cancer
20.	ABX + AC	AC followed by ABX: 260 mg/m^2 vs AC followed by Taxol® Rx length 16 wks	Breast Cancer-Adjuvant	Adjuvant Dose dense Registrational Trial
21.	ABX + AC (+G-CSF)	AC q2wk followed by ABX: 260 mg/m^2 +G-CSF q2wk Rx length 16 wks	Breast Cancer-Adjuvant	Phase II dose dense pilot adjuvant study of Abraxane™ in breast cancer
22.	ABX + AC (+ Avastin®)	Dose dense AC followed by ABX (+ Avastin® in HER2+ pts)	Breast Cancer-Adjuvant	Pilot adjuvant breast cancer study
23.	ABX + AC	AC followed by ABX q2wk or q3wk	Breast Cancer-Adjuvant	BIG study: Dose dense vs standard adjuvant chemotherapy
24.	ABX (ABI-007) + AC + Neulasta®	AC followed by ABX q2wk x 4	Breast Cancer - Adjuvant	Phase II – Pilot Study Evaluating the Safety of a Dose-Dense Regime – AC x 4 => ABI-007 x 4 Q 2 WEEKS + Neulasta® – Given as Adjuvant Chemotherapy of High-Risk Women with Early Breast Cancer
25.	ABX +FEC (+Herceptin®)	ABX: 100 mg/m^2 qwk x 12 followed by 5-FU: 500 mg/m^2 q3wk Epirubicin: 100mg/m^2 (without Herceptin®) or Epirubicin: 75 mg/m^2	Locally Advanced Breast Cancer-Neoadjuvant	A Phase II Study of Neoadjuvant Chemotherapy with Sequential Weekly Nanoparticle Albumin Bound Paclitaxel (Abraxane™) Followed by 5-Fluorouracil, Epirubicin, Cyclophosphamide

Row No.	Combination	Regime/Dosage	Study therapy type	Protocol title
		(with Herceptin® for HER2+ pts) Cyclophosphamide: 500 mg/m^2 q3wk		(FEC) in Locally Advanced Breast Cancer
26.	ABX + Gemcitabine + Epirubicin	Arm 1: Neoadjuvant: Gem: 2000 mg/m^2, ABX: 175 mg/m^2, Epi 50 mg/m^2 q2wk x 6 Arm 2: Adjuvant: Gem: 2000 mg/m^2, ABX: 220 mg/m^2 q2wk x 4	Breast Cancer - Neoadjuvant	Phase II Trial of Dose Dense Neoadjuvant Gemcitabine, Epirubicin, ABI-007 (GEA) in Locally Advanced or Inflammatory Breast Cance
27.	ABX + Herceptin®	ABX: 260 mg/m^2 q2wk + Herceptin® followed by Navelbine® + Herceptin®	Breast Cancer - Neoadjuvant	Phase II Multi-center study neoadjuvant.
28.	ABX + Carboplatin (+ Herceptin®) +AC	TAC vs AC followed by ABX + carbo vs AC followed by ABX + carbo + Herceptin®	Breast Cancer - Neoadjuvant	3 arms Randomized dose dense phase II trial of neoadjuvant chemotherapy in patients with breast cancer
29.	ABX + Capecitabine	ABX: 260 mg/m^2 q3wk x 4 Xeloda® 850 mg/m^2 D1-14 q3wk x 4	Breast Cancer - Neoadjuvant	Phase II neoadjuvant trial of Abraxane™ and capecitabine in locally advanced breast cancer
30.	ABX + Carboplatin (+ Avastin®)	ABX qwk carbo qwk + Avastin® in HER2+ pts	Breast Cancer - Neoadjuvant	Phase I/II trial of neoadjuvant chemotherapy (NCT) with weekly nanoparticle paclitaxel (ABI-007, Abraxane™) in combination with carboplatin and Avastin® in clinical stage I-III.
31.	ABX + Carboplatin + Herceptin® + Avastin®	ABX: 100 mg/m^2 qwk x 3/4 Carbo: AUC = 5 + Herceptin® + Avastin® 4 week cycle x 6	Breast Cancer - Neoadjuvant	Phase II study of weekly bevacizumab administered with weekly trastuzumab, ABI-007, and carboplatin as preoperative therapy in HER2-neu gene amplified breast cancer tumors
32.	ABX + Lapatinib	ABX: 260 mg/m^2 q3wk Lapatinib: 1000 mg/day	Breast Cancer - Neoadjuvant	Pilot neoadjuvant trial with combination of ABI-007 (Abraxane™) and GW572016

Row No.	Combination	Regime/Dosage	Study therapy type	Protocol title
				(Lapatinib)
33.	ABX + Capecitabine	ABX: 200 mg/m^2 q3wk x 4 Xeloda®: 1000 mg/m^2 D1-14 q3wk x 4	Breast Cancer - Neoadjuvant	Phase II neoadjuvant trial of Abraxane™ and capecitabine in locally advanced breast cancer
34.	ABX ± Avastin® + AC (+ G-CSF)	ABX qwk ± Avastin® followed by A qwk + C daily vs Taxol® qwk ± Avastin® followed by A qwk + C daily	Breast Cancer - Neoadjuvant	Phase III trial of paclitaxel vs Abraxane™ with or without Avastin® in combination with doxorubicin and cyclophosphamide plus G-CSF
35.	ABX + AC	ABX followed by AC	Breast Cancer - Neoadjuvant	Phase II neoadjuvant trial with gene expression analyses
36.	ABX + Carboplatin + Avastin®	ABX: 300 mg/m^2 q3wk Carbo: AUC = 6 q3wk Avastin®: 15 mg/kg 4 cycles	1st line Advanced NSCLC	An open label phase II trial of Abraxane™, carboplatin and Avastin® in patients with advanced non-squamous non-small cell lung cancer
37.	ABX + Carboplatin	L1: ABX: 225 mg/m^2 L2: ABX: 260 mg/m^2 L3: ABX: 300 mg/m^2 Cohorts 1-4: ABX q3wk Cohorts 5-7: ABX weekly Cohort 8: 75 additional patients Carbo fixed at AUC = 6 q3wk	Advanced NSCLC	Phase II toxicity pilot study of Abraxane™ and carboplatin in advanced non-small cell lung cancer.
38.	ABX + Carboplatin	Carbo: AUC = 6 + ABX vs Carbo: AUC = 6 + Taxol®: 225 mg/m^2	1st line NSCLC	Phase III Registration - NSCLC 1st line therapy
39.	ABX + Carboplatin	ABX: 100 mg/m^2 d1, 8, 15 Carbo: AUC = 6 q4wk Amendment: ABX: 125 mg/m^2 D1, 8, 15	1st line NSCLC	Phase II Trial of weekly Abraxane™ plus carboplatin in 1st-line NSCLC
40.	ABX + Carboplatin + Avastin®	Weekly	NSCLC	
41.	ABX + Carboplatin	Arm 1: ABX: 100, 125, 150 mg/m^2 D1, 8, 15 q4wk Arm 2: ABX 220, 260, 300, 340 mg/m^2 q3wk	Lung Cancer - Advanced Solid Tumor Malignancy	Phase I Trial of carboplatin and Abraxane™ on a weekly and every three week schedule in

Row No.	Combination	Regime/Dosage	Study therapy type	Protocol title
		Arm 3: ABX 100, 125, 150 mg/m^2 D1, 8 Carbo: AUC = 6 in all arms		patients with Advanced Solid Tumor Malignancies
42.	ABX + Gemcitabine or ABX + Avastin®		NSCLC	Abraxane™ in combination with gemcitabine or Avastin®
43.	ABX + Gemcitabine		NSCLC	Phase I trial of Abraxane™ in combination with gemcitabine
44.	ABX + Carboplatin + Avastin®	ABX: 225, 260, 300 mg/m^2 Carbo: AUC = 6 q3wk + Avastin®	Lung Cancer	Phase I/II study of Abraxane™ and carboplatin AUC 6, plus Avastin® (Standard 3+3 Phase I design; PhII: 40 pts)
45.	ABX + Alimta®	ABX: 220, 260, 300 mg/m^2 q3wk Pemtrexed: 500mg q3wk	Lung Cancer	Phase I/II study of Abraxane™ + Alimta® for 2nd-line NSCLC
46.	ABX + Cisplatin		Lung Cancer	Phase I/II trial of Abraxane™ plus cisplatin in advanced NSCLC
47.	ABX + Navelbine® + Cisplatin		Lung Cancer	Phase I/II study of Abraxane™, Navelbine®, and Cisplatin for treatment of advanced NSCLC
48.	ABX + Carboplatin	ABX: 300 mg/m^2 q3wk Carbo: AUC =6 q3wk	SCLC	Phase II trial of Abraxane™ and carboplatin in extensive stage small cell lung cancer
49.	ABX + Carboplatin	ABX: 100 mg/m^2 qwk x 3/4 Carbo: AUC = 6	Ovarian Cancer	A phase II trial of Abraxane™ + Carboplatin in recurrent ovarian cancer
50.	ABX + Carboplatin	ABX: qwk ABX: q3w Carbo: AUC = 6 both arms	Ovarian Cancer	Phase I study of Abraxane™ plus carbo for treatment of advanced ovarian cancer
51.	ABX + Carboplatin	ABX: TBD by ABI-CA034 vs Taxol® 175 mg/m^2 Carbo: AUC = 6 in both arms	Ovarian Cancer	1st line, optimally debulked, registration trial. Carbo AUC 6 + ABX vs Carbo + Taxol® 175 mg/m^2. Endpoint: relapse free survival, survival

Row No.	Combination	Regime/Dosage	Study therapy type	Protocol title
52.	ABX + Avastin®	ABX: 100 mg/m^2 qwk x 3/4 Avastin®: 10mg/m^2 q2wk	Ovarian Cancer	Phase II study of bevacizumab with Abraxane™ in patients with recurrent, platinum resistant primary epithelial ovarian or primary peritoneal carcinoma
53.	ABX + 5-FU + Cisplatin	ABX: D1 5-FU: 750 mg/m^2 CIV x 5 cisplatin: 75 mg/m^2 D1 followed by XRT/surgery	Head and Neck Cancer	Unresectable localized head and neck cancer Phase II Abraxane™ in combination with 5-FU and cisplatin
54.	ABX + 5-FU + Cisplatin	5-FU: 750 mg/m^2 CIV x 5 cisplatin: 75 mg/m^2 D1 ± ABX D1 followed by XRT/surgery	Head and Neck Cancer	Unresectable localized head and neck cancer Phase III 5-FU and cisplatin with or without Abraxane™
55.	ABX + Cetuximab		Head and Neck Cancer	Phase II multicenter trial of Abraxane™ in combination with cetuximab in 1st line treatment of locally advanced or metastatic head and neck cancer
56.	ABX + Rapamycin	ABX: 100mg/m^2 qwk Rapamycin: 5-40 mg dose escalation	Solid Tumors	Phase I Study of Rapamycin in Combination with Abraxane™ in Advanced Solid Tumors
57.	ABX + Satraplatin		Solid Tumors	Phase I trial of Abraxane™ and Satraplatin
58.	ABX + Gemcitabine	ABX: 180, 220, 260, 300, 340 mg/m^2 q3wk Gemcitabine: 1000mg/m^2 D1 and D8	Advanced Solid Tumors	Phase I Trial of Abraxane™ in combination with Gemcitabine
59.	ABX + Gefitinib	ABX: 100 mg/m^2 qwk x 3/4 Gefitinib starting at 1000 mg/d x 2	Advanced Solid Tumors	Phase I dose escalation study of gefitinib chemosensitization pulse given prior to weekly Abraxane™
60.	ABX + Avastin®		Metastatic Melanoma	Phase II study of Abraxane™ and Avastin® in metastatic melanoma
61.	ABX + Avastin®		Melanoma	Abraxane™ and Avastin® as therapy for patients with malignant melanoma

Row No.	Combination	Regime/Dosage	Study therapy type	Protocol title
62.	ABX + Carboplatin		Metastatic Melanoma	Phase II study of Abraxane™ and carboplatin in metastatic melanoma
63.	ABX + Sorafenib + Carboplatin	ABX: qwk Sorafenib: D2-19 Carbo: AUC = 6 D1	Metastatic Melanoma	Phase II study of Abraxane™ in combination with carboplatin and sorafenib in metastatic melanoma
64.	ABX + Capecitabine		Metastatic Colorectal Cancer (after failure of oxaliplatin-based therapy and irinotecan-based therapy)	Phase II trial of Abraxane™ in combination with Xeloda® for previously treated patient with advance or metastatic colorectal cancer
65.	ABX + Gemcitabine	Weekly	Pancreatic Cancer	Phase I study of Abraxane™ in combination with gemcitabine in pancreatic cancer
66.	ABX + Gemcitabine	ABX + Gem vs Gem	Pancreatic Cancer	Phase III registration trial in pancreatic cancer
67.	ABX + anti-angiogenic agents			Abraxane™ combined with anti-angiogenic agents, e.g. Avastin®
68.	ABX + proteasome inhibitors			Abraxane™ combined with proteasome inhibitors, e.g. Velcade®
69.	ABX + EGFR inhibitors			Abraxane™ combined with EGFR inhibitors, e.g. Tarceva®

[0119] As used in herein (for example in Table 1), ABX refers to Abraxane™; GW572016 refers to lapatinib; Xel refers to capecitabine or Xeloda®; bevacizumab is also known as Avastin®; trastuzumab is also known as Herceptin®; pemtrexed is also known as Alimta®; cetuximab is also known as Erbitux®; gefitinib is also known as Iressa®; FEC refers to a combination of 5-fluorouracil, Epirubicin and Cyclophosphamide; AC refers to a combination of Adriamycin plus Cyclophosphamide; TAC refers to a FDA approved adjuvant breast cancer regime; RAD001 refers to a derivative of rapamycin; NSCLC refers to non-small cell lung cancer; and SCLC refers to small cell lung cancer.

[0120] As used herein (for example in Table 1), AUC refers to area under curve; q4wk refers to a dose every 4 weeks; q3wk refers to a dose every 3 weeks; q2wk refers to a dose every 2 weeks; qwk refers to a weekly dose; qwk x 3/4 refers to a weekly dose for 3 weeks with the 4th week off; qwk x 2/3 refers to a weekly dose for 2 weeks with the 3rd week off.

Combination therapy with radiation therapy and surgery

[0121] In another aspect, the present invention provides a method of treating proliferative disease (such as cancer) comprising a first therapy comprising administering a taxane (particularly nanoparticles comprising a taxane) and a carrier protein and a second therapy comprising radiation and/or surgery.

[0122] In some embodiments, the method comprises: a) a first therapy comprising administering to the individual a composition comprising nanoparticles comprising an effective amount of a taxane and a carrier protein (such as albumin) and b) a second therapy comprising radiation therapy, surgery, or combinations thereof. In some embodiments, the taxane is coated with the carrier protein (such as albumin). In some embodiments, the second therapy is radiation therapy. In some embodiments, the second therapy is surgery.

[0123] In some embodiments, the method comprises a) a first therapy comprising administering to the individual a composition comprising nanoparticles comprising paclitaxel and an albumin; and b) a second therapy comprising radiation therapy, surgery, or combinations thereof. In some embodiments, the second therapy is radiation therapy. In some embodiments, the second therapy is surgery. In some embodiments, the paclitaxel/albumin nanoparticles have an average diameter of no greater than about 200 nm. In some embodiments, the paclitaxel/albumin nanoparticle composition is substantially free (such as free) of surfactant (such as Cremophor). In some embodiments, the weight ratio of the albumin to paclitaxel in the composition is about 18:1 or less, such as about 9:1 or less. In some embodiments, the paclitaxel is coated with albumin. In some embodiments, the paclitaxel/albumin nanoparticles have an average diameter of no greater than about 200 nm and the paclitaxel/albumin composition is substantially free (such as free) of surfactant (such as Cremophor). In some embodiments, the paclitaxel/albumin nanoparticles have an average diameter of no greater than about 200 nm and the paclitaxel is coated with albumin. In some embodiments, the nanoparticle composition is Abraxane^{TM}.

[0124] The administration of the nanoparticle composition may be prior to the radiation and/or surgery, after the radiation and/or surgery, or concurrent with the radiation and/or surgery. For example, the administration of the nanoparticle composition may precede or follow the radiation and/or surgery therapy by intervals ranging from minutes to weeks. In some embodiments, the time period between the first and the second therapy is such that the taxane and the radiation/surgery would still be able to exert an advantageously combined effect on the cell. For example, the taxane (such as paclitaxel) in the nanoparticle composition may be administered less than about any of 1, 3, 6, 9, 12, 18, 24, 48, 60, 72, 84, 96, 108, 120 hours prior to the radiation and/or surgery. In some embodiments, the nanoparticle composition is administered less than about 9 hours prior to the radiation and/surgery. In some embodiments, the nanoparticle composition is administered less than about any of 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 days prior to the radiation/surgery. In some embodiments, the taxane (such as paclitaxel) in the nanoparticle composition is administered less than about any of 1, 3, 6, 9, 12, 18, 24, 48, 60, 72, 84, 96, 108, or 120 hours after the radiation and/or surgery. In some embodiments, it may be desirable to extend the time period for treatment significantly, where several days to several weeks lapse between the two therapies.

[0125] Radiation contemplated herein includes, for example, γ-rays, X-rays (external beam), and the directed delivery of radioisotopes to tumor cells. Other forms of DNA damaging factors are also contemplated such as microwaves and UV irradiation are also contemplated. Radiation may be given in a single dose or in a series of small doses in a dose-fractionated schedule. The amount of radiation contemplated herein ranges from about 1 to about 100 Gy, including, for example, about 5 to about 80, about 10 to about 50 Gy, or about 10 Gy. The total dose may be applied in a fractioned regime. For example, the regime may comprise fractionated individual doses of 2 Gy. Dosage ranges for radioisotopes vary widely, and depends on the half-life of the isotope and the strength and type of radiation emitted.

[0126] When the radiation comprises use of radioactive isotopes, the isotope may be conjugated to a targeting agent, such as a therapeutic antibody, which carries the radionucleotide to the target tissue. Suitable radioactive isotopes include, but are not limited to, astatine^{211}, ^{14}carbon, ^{51}chromium, ^{36}chlorine, ^{57}iron, ^{58}cobalt, copper^{67}, ^{152}Eu, gallium^{67}, ^{3}hydrogen, iodine^{123}, iodine^{131}, indium^{111}, ^{59}ion, ^{32}phosphorus, rhenium^{186}, ^{75}selenium, ^{35}sulphur, technicium^{99m}, and/or yttrium^{90}.

[0127] In some embodiments, enough radiation is applied to the individual so as to allow reduction of the normal dose of the taxane (such as paclitaxel) in the nanoparticle composition required to effect the same degree of treatment by at least about any of 5%, 10%, 20%, 30%, 50%, 60%, 70%, 80%, 90%, or more. In some embodiments, enough taxane in the nanoparticle composition is administered so as to allow reduction of the normal dose of the radiation required to effect the same degree of treatment by at least about any of 5%, 10%, 20%, 30%, 50%, 60%, 70%, 80%, 90%, or more. In some embodiments, the dose of both the taxane (such as paclitaxel) in the nanoparticle composition and the radiation are reduced as compared to the corresponding normal dose of each when used alone.

[0128] In some embodiments, the combination of administration of the nanoparticle composition and the radiation therapy produce supra-additive effect. In some embodiments, the taxane (such as paclitaxel) in the nanoparticle composition is administered once at the dose of 90 mg/kg, and the radiation is applied five times at 80 Gy daily.

[0129] Surgery described herein includes resection in which all or part of cancerous tissue is physically removed, exercised, and/or destroyed. Tumor resection refers to physical removal of at least part of a tumor. In addition to tumor resection, treatment by surgery includes laser surgery, cryosurgery, electrosurgery, and micropically controlled surgery (Mohs surgery). Removal of superficial surgery, precancers, or normal tissues are also contemplated.

[0130] The radiation therapy and/or surgery may be carried out in addition to the administration of chemotherapeutic agents. For example, the individual may first be administered with a taxane-containing nanoparticle composition and at least one other chemotherapeutic agent, and subsequently be subject to radiation therapy and/or surgery. Alternatively, the individual may first be treated with radiation therapy and/or surgery, which is then followed by the administration of a nanoparticle composition and at least one other chemotherapeutic agent. Other combinations are also contemplated.

[0131] Administration of nanoparticle compositions disclosed above in conjunction with administration of chemotherapeutic agent is equally applicable to those in conjunction with radiation therapy and/or surgery.

[0132] In some embodiments, the nanoparticle composition of the taxane and/or the chemotherapeutic agent is administered in conjunction with radiation according to any of the dosing regimes described in Table 2.

[0133] In some embodiments, there is provided a method of treating NSCLC in an individual comprises a) a first therapy comprising administering to the individual a composition comprising nanoparticles comprising taxane (such as paclitaxel) and an albumin; and b) a second therapy comprising radiation as provided in Rows 1 to 5 in Table 2. In some embodiments, the administration of the nanoparticle composition and the chemotherapeutic agent may be any of the dosing regimes as indicated in Rows 1 to 5 in Table 2.

[0134] In some embodiments, there is provided a method of treating head and neck cancer in an individual comprises a) a first therapy comprising administering to the individual a composition comprising nanoparticles comprising taxane (such as paclitaxel) and an albumin; and b) a second therapy comprising radiation as provided in Rows 6 to 9 in Table 2. In some embodiments, the administration of the nanoparticle composition and the chemotherapeutic agent may be any of the dosing regimes as indicated in Rows 6 to 9 in Table 2.

[0135] In some embodiments, there is provided a method of treating pancreatic cancer in an individual comprises a) a first therapy comprising administering to the individual a composition comprising nanoparticles comprising taxane (such as paclitaxel) and an albumin; and b) a second therapy comprising radiation as provided in Row 10 in Table 2. In some embodiments, the administration of the nanoparticle composition and the chemotherapeutic agent may be the dosing regimes as indicated in Row 10 in Table 2.

[0136] In some embodiments, there is provided a method of treating gastric malignancies in an individual comprises a) a first therapy comprising administering to the individual a composition comprising nanoparticles comprising taxane (such as paclitaxel) and an albumin; and b) a second therapy comprising radiation as provided in Row 11 in Table 2. In some embodiments, the administration of the nanoparticle composition and the chemotherapeutic agent may be the dosing regimes as indicated in Row11 in Table 2.

TABLE 2

Row No.	Combination	Regime/Dosage	Study therapy type	Protocol title
1	ABX + Radiation		NSCLC	Phase I/II trial of Abraxane™ combined with radiation
2	ABX + Carboplatin + Radiation		NSCLC	Phase I/II trial of Abraxane™ and carboplatin combined with radiation.
3	ABX +	1 cycle ABX/Carbo induction	NSCLC	Phase II chemoradiation

Row No.	Combination	Regime/Dosage	Study therapy type	Protocol title
	Carboplatin + Radiation	followed by 2 or 3 times weekly pulse ABX + radiation		in NSCLC
4	ABX + Carboplatin + Radiation		NSCLC	Abraxane™ /carboplatin induction followed by Abraxane™ + radiation in stage III A&B PS2 NSCLC patients
5	ABX + Carboplatin + Radiation	ABX qwk + carbo + radiation followed by ABX q3wk + carbo	NSCLC	Phase II study
6	ABX + Radiation		Head and Neck Cancer	Abraxane™ as a radiosensitizer in head and neck cancer
7	ABX + Cetuximab + Radiation		Head and Neck Cancer	PhaseI/II Abraxane™ in combination with cetuximab and radiation
8	ABX + Carboplatin + 5-FU + Hydroxyurea + Radiation	Induction: ABX 135 mg/m^2 qwk + carbo: AUC = 2 followed by Concurrent chemoradiation: ABX: 100 mg/m^2 5-FU: 600 mg/m^2 hydroxyurea: 5000 mg BID	Head and Neck Cancer	Phase I/II study of induction chemotherapy with Abraxane™ and carboplatin followed by concomitant fluorouracil, hydroxyurea, Abraxane™ and IMRT for locally advanced head and neck cancers
9	ABX + Carboplatin + Erbitux® + Radiation	ABX: 20-50 mg/m^2 qwk x 7 dose escalation Eribitux®: 400 mg/m^2 day 7, 250 mg/m^2 qwk x 7 Carbo: AUC = 1.5 qwk x 7 IMRT	Locally Advanced Head and Neck Cancer	Phase I trial of Abraxane™ in combination with carboplatin, cetuximab and IMRT in locally advanced squamous cell cancer of the head and neck
10	ABX + Gemcitabine + Radiation	qwk	Pancreatic Cancer	A randomized phase II trial of weekly gemcitabine, Abraxane™, and external irradiation for locally advanced pancreatic cancer
11	ABX + Cisplatin + Radiation		Gastric Malignancies	Phase I/II combination of Abraxane™/cisplatin and radiation for patients with resected gastric/GEJ malignancies.

[0137] In some embodiments, the invention provides pharmaceutical compositions comprising nanoparticles comprising a taxane (such as paclitaxel) and a carrier protein

(such as albumin) for use in the treatment of a proliferative disease (such as cancer), wherein said use comprises a second therapy comprising radiation therapy, surgery, or combinations thereof.

Metronomic therapy

[0138] The invention also provides metronomic therapy regime. There is provided a method of administering to an individual a composition comprising nanoparticles comprising a taxane (such as paclitaxel, docetaxel, or ortataxel) and a carrier protein (such as albumin) based on a metronomic dosing regime. The methods are applicable to methods of treatment, delaying development, and other clinical settings and configurations described herein. For example, in some embodiments, the methods are useful for treatment of proliferative diseases (such as cancer).

[0139] "Metronomic dosing regime" used herein refers to frequent administration of a taxane at without prolonged breaks at a dose below the established maximum tolerated dose via a traditional schedule with breaks (hereinafter also referred to as a "standard MTD schedule" or a "standard MTD regime"). In metronomic dosing, the same, lower, or higher cumulative dose over a certain time period as would be administered via a standard MTD schedule may ultimately be administered. In some cases, this is achieved by extending the time frame and/or frequency during which the dosing regime is conducted while decreasing the amount administered at each dose. Generally, the taxane administered via the metronomic dosing regime of the present invention is better tolerated by the individual. Metronomic dosing can also be referred to as maintenance dosing or chronic dosing.

[0140] In some embodiments, there is provided a method of administering a composition comprising nanoparticles comprising a taxane and a carrier protein (such as albumin), wherein the nanoparticle composition is administered over a period of at least one month, wherein the interval between each administration is no more than about a week, and wherein the dose of the taxane at each administration is about 0.25% to about 25% of its maximum tolerated dose following a traditional dosing regime. In some embodiments, there is provided a method of administering a composition comprising nanoparticles comprising paclitaxel and an albumin, wherein the nanoparticle composition is administered over a period of at least one month, wherein the interval between each administration is no more than about a week, and wherein the dose of the taxane at each administration is about 0.25% to about 25% of its maximum tolerated dose following a traditional dosing regime.

[0141] In some embodiments, the dosing of the taxane (such as paclitaxel) in the nanoparticle composition per administration is less than about any of 1%, 2%, 3&, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 18%, 20%, 22%, 24%, or 25% of the MTD for the same taxane (such as paclitaxel) in the same formulation following a given traditional dosing schedule. Traditional dosing schedule refers to the dosing schedule that is generally established in a clinical setting. For example, the tradition dosing schedule for Abraxane™ is a three-weekly schedule, i.e., administering the composition every three weeks.

[0142] In some embodiments, the dosing of the taxane (such as paclitaxel) per administration is between about 0.25% to about 25% of the corresponding MTD value, including for example any of about 0.25% to about 20%, about 0.25% to about 15%, about 0.25% to about 10%, about 0.25% to about 20%, and about 0.25% to about 25%, of the corresponding MTD value. The MTD value for a taxane following a traditional dosing schedule is known or can be easily determined by a person skilled in the art. For example, the MTD value when Abraxane™ is administered following a traditional three-week dosing schedule is about 300 mg/m^2.

[0143] In some embodiments, there is provided a method of administering a composition comprising nanoparticles comprising a taxane and a carrier protein (such as albumin), wherein the nanoparticle composition is administered over a period of at least one month, wherein the interval between each administration is no more than about a week, and wherein the dose of the taxane at each administration is about 0.25 mg/m^2 to about 25 mg/m^2. In some embodiments, there is provided a method of administering a composition comprising nanoparticles comprising paclitaxel and an albumin, wherein the nanoparticle composition is administered over a period of at least one month, wherein the interval between each administration is no more than about a week, and wherein the dose of the taxane at each administration is about 0.25 mg/m^2 to about 25 mg/m^2.

[0144] In some embodiments, the dose of the taxane (such as paclitaxel) at each administration is less than about any of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 18, 20, 22, 25, and 30 mg/m^2. For example, the dose of the taxane (such as paclitaxel) can range from about 0.25 mg/m^2 to about 30 mg/m^2, about 0.25 mg/m^2 to about 25 mg/m^2, about 0.25 mg/m^2 to about 15 mg/m^2, about 0.25 mg/m^2 to about 10 mg/m^2, and about 0.25 mg/m^2 to about 5 mg/m^2.

[0145] Dosing frequency for the taxane (such as paclitaxel) in the nanoparticle composition includes, but is not limited to, at least about any of once a week, twice a week,

three times a week, four times a week, five times a week, six times a week, or daily. Typically, the interval between each administration is less than about a week, such as less than about any of 6, 5, 4, 3, 2, or 1 day. In some embodiments, the interval between each administration is constant. For example, the administration can be carried out daily, every two days, every three days, every four days, every five days, or weekly. In some embodiments, the administration can be carried out twice daily, three times daily, or more frequent.

[0146] The metronomic dosing regimes described herein can be extended over an extended period of time, such as from about a month up to about three years. For example, the dosing regime can be extended over a period of any of about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 18, 24, 30, and 36 months. Generally, there are no breaks in the dosing schedule.

[0147] The cumulative dose of the taxane (such as paclitaxel) administered by the metronomic regime may be higher than that of the taxane administered according to a standard MTD dosing schedule over the same time period. In some embodiments, the cumulative dose of the taxane administered by the metronomic regime equals to or is lower than that of the taxane administered according to a standard MTD dosing schedule over the same time period.

[0148] It is understood that the teaching provided herein is for examples only, and that metronomic dosing regime can be routinely designed in accordance with the teachings provided herein and based upon the individual standard MTD schedule, and that the metronomic dosing regime used in these experiments merely serves as one example of possible changes in dosing interval and duration which are made to a standard MTD schedule to arrive at an optimal metronomic dosing regime.

[0149] The metronomic dosing regime described herein may be used alone as a treatment of a proliferative disease, or carried out in a combination therapy context, such as the combination therapies described herein. In some embodiments, the metronomic therapy dosing regime may be used in combination or conjunction with other established therapies administered via standard MTD regimes. By "combination or in conjunction with" it is meant that the metronomic dosing regime of the present invention is conducted either at the same time as the standard MTD regime of established therapies, or between courses of induction therapy to sustain the benefit accrued to the individual by the induction therapy, the intent is to continue to inhibit tumor growth while not unduly compromising the individual's health or the individual's ability to withstand the next

course of induction therapy. For example, a metronomic dosing regime may be adopted after an initial short course of MTD chemotherapy.

[0150] The nanoparticle compositions administered based on the metronomic dosing regime described herein can be administered to an individual (such as human) via various routes, such as parenterally, including intravenous, intra-arterial, intrapulmonary, oral, inhalation, intravesicular, intramuscular, intra-tracheal, subcutaneous, intraocular, intrathecal, or transdermal. For example, the nanoparticle composition can be administered by inhalation to treat conditions of the respiratory tract. The composition can be used to treat respiratory conditions such as pulmonary fibrosis, broncheolitis obliterans, lung cancer, bronchoalveolar carcinoma, and the like. In some embodiments, the nanoparticle composition is administered orally.

[0151] Some various exemplary embodiments are provided below.

[0152] In some embodiments, there is provided a method of administering a composition comprising nanoparticles comprising a taxane and a carrier protein (such as albumin), wherein the nanoparticle composition is administered over a period of at least one month, wherein the interval between each administration is no more than about a week, and wherein the dose of the taxane at each administration is about 0.25% to about 25% of its maximum tolerated dose following a traditional dosing regime. In some embodiments, the taxane is coated with the carrier protein (such as albumin). In some embodiments, the dose of the taxane per administration is less than about any of 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 18%, 20%, 22%, 24%, or 25% of the maximum tolerated dose. In some embodiments, the taxane is administered at least about any of 1x, 2x, 3x, 4x, 5x, 6x, 7x (i.e., daily) a week. In some embodiments, the intervals between each administration are less than about any of 7 days, 6 days, 5 days, 4 days, 3 days, 2 days, and 1 day. In some embodiments, the taxane is administered over a period of at least about any of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 18, 24, 30 and 36 months.

[0153] In some embodiments, there is provided a method of administering a composition comprising nanoparticles comprising paclitaxel and an albumin, wherein the nanoparticle composition is administered over a period of at least one month, wherein the interval between each administration is no more than about a week, and wherein the dose of the taxane at each administration is about 0.25% to about 25% of its maximum tolerated dose following a traditional dosing regime. In some embodiments, the paclitaxel/albumin nanoparticles have an average diameter of no greater than about 200 nm. In some embodiments, the paclitaxel/albumin nanoparticle composition is substantially free (such as

free) of surfactant (such as Cremophor). In some embodiments, the weight ratio of the albumin to paclitaxel in the composition is about 18:1 or less, such as about 9:1 or less. In some embodiments, the paclitaxel is coated with albumin. In some embodiments, the paclitaxel/albumin nanoparticles have an average diameter of no greater than about 200 nm and the paclitaxel/albumin composition is substantially free (such as free) of surfactant (such as Cremophor). In some embodiments, the paclitaxel/albumin nanoparticles have an average diameter of no greater than about 200 nm and the paclitaxel is coated with albumin. In some embodiments, the nanoparticle composition is Abraxane^{TM}.

[0154] In some embodiments, there is provided a method of administering a composition comprising nanoparticles comprising a taxane and a carrier protein (such as albumin), wherein the nanoparticle composition is administered over a period of at least one month, wherein the interval between each administration is no more than about a week, and wherein the dose of the taxane at each administration is about 0.25 mg/m^2 to about 25 mg/m^2. In some embodiments, the taxane is coated with the carrier protein (such as albumin). In some embodiments, the dose of the taxane per administration is less than about any of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 18, 20, 22, and 25 mg/m^2. In some embodiments, the taxane is administered at least about any of 1x, 2x, 3x, 4x, 5x, 6x, 7x (i.e., daily) a week. In some embodiments, the intervals between each administration are less than about any of 7 days, 6 days, 5 days, 4 days, 3 days, 2 days, and 1 day. In some embodiments, the taxane is administered over a period of at least about any of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 18, 24, 30 and 36 months.

[0155] In some embodiments, there is provided a method of administering a composition comprising nanoparticles comprising paclitaxel and an albumin, wherein the nanoparticle composition is administered over a period of at least one month, wherein the interval between each administration is no more than about a week, and wherein the dose of the taxane at each administration is about 0.25 mg/m^2 to about 25 mg/m^2. In some embodiments, the paclitaxel/albumin nanoparticles have an average diameter of no greater than about 200 nm. In some embodiments, the paclitaxel/albumin nanoparticle composition is substantially free (such as free) of surfactant (such as Cremophor). In some embodiments, the weight ratio of the albumin to paclitaxel in the composition is about 18:1 or less, such as about 9:1 or less. In some embodiments, the paclitaxel is coated with albumin. In some embodiments, the paclitaxel/albumin nanoparticles have an average diameter of no greater than about 200 nm and the paclitaxel/albumin composition is substantially free (such as free) of surfactant (such as Cremophor). In some embodiments,

the paclitaxel/albumin nanoparticles have an average diameter of no greater than about 200 nm and the paclitaxel is coated with albumin. In some embodiments, the nanoparticle composition is Abraxane™.

[0156] In some embodiments, the Abraxane™ (or other paclitaxel/albumin nanoparticle compositions) is administered at the dose of about 3 mg/kg to about 10 mg/kg daily. In some embodiments, the Abraxane™ is administered at the dose of about 6 mg/kg to about 10 mg/kg daily. In some embodiments, the Abraxane™ is administered at the dose of about 6 mg/kg daily. In some embodiments, Abraxane™ is administered at the dose of about 3 mg/kg daily.

[0157] The invention also provides compositions for use in the metronomic regime(s) described herein. In some embodiments, there is provided a composition comprising nanoparticles comprising a taxane and a carrier protein (such as albumin), wherein said composition is administered to an individual via a metronomic dosing regime, such as the dosing regime described herein.

Other aspects of the invention

[0158] In another aspects, there are provided methods of treating proliferative diseases comprising administering a composition comprising nanoparticles comprising a taxane (including pacltiaxel, docetaxel, or ortataxel) and a carrier protein (such as albumin). In some embodiments, there is provided a method of treating cancer comprising administering a composition comprising nanoparticles comprising ortataxel and a carrier protein (such as albumin).

[0159] In some embodiments, there is provided methods of treating proliferative diseases comprising administering a composition comprising nanoparticles comprising a thiocolchicine or its derivative (such as dimeric thiocolchicine) and a carrier protein (such as albumin). In some embodiments, there is provided a method of treating cancer comprising administering a composition comprising nanoparticles comprising dimeric colchicines and a carrier protein (such as albumin). In some embodiments, the nanoparticle composition is any of (and in some embodiments selected from the group consisting of) Nab-5404, Nab-5800, and Nab-5801.

[0160] In some embodiments, there is provided a method of treating cancer comprising administering a composition comprising nanoparticles comprising paclitaxel, wherein the nanoparticle composition is administered according to any of the dosing

regimes described in Table 3. In some embodiments, the cancer is a Taxane refractory metastatic breast cancer.

TABLE 3

Row No.	Combination	Regimen/Dosage	Study therapy type	Protocol title
1.	ABX alone	ABX: 125 mg/m^2 qwk x 3/4	Metastatic Breast Cancer	Phase II study with weekly Abraxane™ treatment in taxane-refractory MBC patients
2.	ABX alone	Arm 1: ABX 130 mg/m^2 qwk Arm 2: ABX 260 mg/m^2 q2wk Arm 3: ABX 260 mg/m^2 q3wk	Metastatic Breast Cancer	3-arm phase II trial in 1st-line Her-2- MBC patients.
3.	ABX alone (Capxol)	ABX: 260 mg/m^2 q3wk vs Taxol: 175 mg/m^2 q3wk	Metastatic Breast Cancer	Phase II Controlled, Randomized, Open Label Study to Evaluate the Efficacy and Safety of Capxol (a Cremophor-Free Nanoparticle Paclitaxel) and cremophor-formulated paclitaxel injection in Patient with Metastatic Breast Cancer
4.	ABX alone	Arm 1: ABX weekly Arm 2: ABX q3wk Arm 3: Taxol weekly	Metastatic Breast Cancer	3-arm phase II trial in 1st-line and 2nd-line MBC, with biological correlates analysis
5.	ABX alone	ABX: 300 mg/m^2 q3wk	Stage IIA, IIB, IIIA, IIIB and IV breast cancer	Phase II trial of neoadjuvant chemotherapy (NCT) with nanoparticle paclitaxel (ABI-007, Abraxane) in women with clinical stage IIA, IIB, IIIA, IIIB and IV (with intact primary) breast cancers
6.	ABX alone	ABX: 125 mg/m^2 qwk x 3/4	1st-line advanced NSCLC	Phase I/II study of Abraxane monotherapy in 1st-line advanced NSCLC
7.	ABX alone	ABX 260 mg/m^2 q3wk	1st-line NSCLC	Phase II ABX mono in 1st-line NSCLC
8.	ABX alone	Arm 1: ABX q3wk Arm 2: ABX qwk Doses TBD	2nd line NSCLC	Phase II study of Abraxane monotherapy in 2nd-line NSCLC
9.	ABX alone	ABX: 100mg/m^2 qwk vs ABX: 260 mg/m^2 q3wk	Prostate Cancer	Randomized phase II study Abraxane™ weekly vs every three weeks in front line HRP
10.	ABX alone	ABX qwk	Prostate Cancer	Phase II ABX in 1st-line prostate cancer
11.	ABX alone	ABX: 150 mg/m^2 qwk x 3/4 for 2 cycles	Prostate Cancer	Phase II neoadjuvant study
12.	ABX alone	ABX: 100 mg/m^2 qwk (no break)	Prostate Cancer	Phase II ABX 100 mg

Row No.	Combination	Regimen/Dosage	Study therapy type	Protocol title
				weekly no break
13.	ABX alone	ABX: 100 mg/m^2 (previously treated) ABX: 150 mg/m^2 (untreated) qwk x 3/4	Malignant Melanoma	Phase II previously treated and untreated metastatic melanoma patients
14.	ABX alone	ABX: 125 mg/m^2 qwk x 3/4	Carcinoma of the cervix	Phase II study of ABX in treatment of persistent or recurrent carcinoma of the cervix
15.	ABX alone		Ovarian Cancer	Phase II study of Abraxane for treatment of advanced ovarian cancer (3rd line)
16.	ABX alone (ABI-007)		non-hematologic malignancies	Phase II single treatment use of ABI-007 for the treatment of non-hematologic malignancies. Compassionate use

Nanoparticle compositions

[0161] The nanoparticle compositions described herein comprise nanoparticles comprising (in various embodiments consisting essentially of) a taxane (such as paclitaxel) and a carrier protein (such as albumin). Nanoparticles of poorly water soluble drugs (such as taxane) have been disclosed in, for example, U.S. Pat. Nos. 5,916,596; 6,506,405; and 6,537,579 and also in U.S. Pat. Pub. No. 2005/0004002A1. Although the description provided below is specific to taxane, it is understood that the same applies to other drugs, such as rapamycin, 17-AAG, and dimeric thiocolchicine.

[0162] In some embodiments, the composition comprises nanoparticles with an average or mean diameter of no greater than about 1000 nanometers (nm), such as no greater than about any of 900, 800, 700, 600, 500, 400, 300, 200, and 100 nm. In some embodiments, the average or mean diameters of the nanoparticles is no greater than about 200 nm. In some embodiments, the average or mean diameters of the nanoparticles is no greater than about 150 nm. In some embodiments, the average or mean diameters of the nanoparticles is no greater than about 100 nm. In some embodiments, the average or mean diameter of the nanoparticles is about 20 to about 400 nm. In some embodiments, the average or mean diameter of the nanoparticles is about 40 to about 200 nm. In some embodiments, the nanoparticles are sterile-filterable.

[0163] The nanoparticles described herein may be present in a dry formulation (such as lyophilized composition) or suspended in a biocompatible medium. Suitable biocompatible media include, but are not limited to, water, buffered aqueous media, saline,

buffered saline, optionally buffered solutions of amino acids, optionally buffered solutions of proteins, optionally buffered solutions of sugars, optionally buffered solutions of vitamins, optionally buffered solutions of synthetic polymers, lipid-containing emulsions, and the like.

[0164] The term "proteins" refers to polypeptides or polymers of amino acids of any length (including full length or fragments), which may be linear or branched, comprise modified amino acids, and/or be interrupted by non-amino acids. The term also encompasses an amino acid polymer that has been modified naturally or by intervention; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification. Also included within this term are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids, etc.), as well as other modifications known in the art. The proteins described herein may be naturally occurring, i.e., obtained or derived from a natural source (such as blood), or synthesized (such as chemically synthesized or by synthesized by recombinant DNA techniques).

[0165] Examples of suitable carrier proteins include proteins normally found in blood or plasma, which include, but are not limited to, albumin, immunoglobulin including IgA, lipoproteins, apolipoprotein B, alpha-acid glycoprotein, beta-2-macroglobulin, thyroglobulin, transferin, fibronectin, factor VII, factor VIII, factor IX, factor X, and the like. In some embodiments, the carrier protein is non-blood protein, such as casein, α-lactalbumin, and β-lactoglobulin. The carrier proteins may either be natural in origin or synthetically prepared. In some embodiments, the pharmaceutically acceptable carrier comprises albumin, such as human serum albumin. Human serum albumin (HSA) is a highly soluble globular protein of M_r 65K and consists of 585 amino acids. HSA is the most abundant protein in the plasma and accounts for 70-80 % of the colloid osmotic pressure of human plasma. The amino acid sequence of HSA contains a total of 17 disulphide bridges, one free thiol (Cys 34), and a single tryptophan (Trp 214). Intravenous use of HSA solution has been indicated for the prevention and treatment of hypovolumic shock (see, e.g., Tullis, *JAMA, 237*, 355-360, 460-463, (1977)) and Houser et al., *Surgery, Gynecology and Obstetrics, 150*, 811-816 (1980)) and in conjunction with exchange transfusion in the treatment of neonatal hyperbilirubinemia (see, e.g., Finlayson, *Seminars in Thrombosis and Hemostasis, 6*, 85-120, (1980)). Other albumins are contemplated, such as bovine serum albumin. Use of such non-human albumins could be appropriate, for

example, in the context of use of these compositions in non-human mammals, such as the veterinary (including domestic pets and agricultural context).

[0166] Human serum albumin (HSA) has multiple hydrophobic binding sites (a total of eight for fatty acids, an endogenous ligand of HSA) and binds a diverse set of taxanes, especially neutral and negatively charged hydrophobic compounds (Goodman et al., *The Pharmacological Basis of Therapeutics*, 9th ed, McGraw-Hill New York (1996)). Two high affinity binding sites have been proposed in subdomains IIA and IIIA of HSA, which are highly elongated hydrophobic pockets with charged lysine and arginine residues near the surface which function as attachment points for polar ligand features (see, e.g., Fehske et al., *Biochem. Pharmcol.*, *30*, 687-92 (198a), Vorum, *Dan. Med. Bull.*, *46*, 379-99 (1999), Kragh-Hansen, *Dan. Med. Bull.*, *1441*, 131-40 (1990), Curry et al., *Nat. Struct. Biol.*, *5*, 827-35 (1998), Sugio et al., *Protein. Eng.*, *12*, 439-46 (1999), He et al., *Nature*, *358*, 209-15 (199b), and Carter et al., *Adv. Protein. Chem.*, *45*, 153-203 (1994)). Paclitaxel and propofol have been shown to bind HSA (see, e.g., Paal et al., *Eur. J. Biochem.*, *268(7)*, 2187-91 (200a), Purcell et al., *Biochim. Biophys. Acta*, *1478(a)*, 61-8 (2000), Altmayer et al., *Arzneimittelforschung*, *45*, 1053-6 (1995), and Garrido et al., *Rev. Esp. Anestestiol. Reanim.*, *41*, 308-12 (1994)). In addition, docetaxel has been shown to bind to human plasma proteins (see, e.g., Urien et al., *Invest. New Drugs*, *14(b)*, 147-51 (1996)).

[0167] The carrier protein (such as albumin) in the composition generally serves as a carrier for the taxane, i.e., the carrier protein in the composition makes the taxane more readily suspendable in an aqueous medium or helps maintain the suspension as compared to compositions not comprising a carrier protein. This can avoid the use of toxic solvents (or surfactants) for solubilizing the taxane, and thereby can reduce one or more side effects of administration of the taxane into an individual (such as a human). Thus, in some embodiments, the composition described herein is substantially free (such as free) of surfactants, such as Cremophor (including Cremophor EL® (BASF)). In some embodiments, the nanoparticle composition is substantially free (such as free) of surfactants. A composition is "substantially free of Cremophor" or "substantially free of surfactant" if the amount of Cremophor or surfactant in the composition is not sufficient to cause one or more side effect(s) in an individual when the nanoparticle composition is administered to the individual.

[0168] The amount of carrier protein in the composition described herein will vary depending on other components in the composition. In some embodiments, the composition comprises a carrier protein in an amount that is sufficient to stabilize the

taxane in an aqueous suspension, for example, in the form of a stable colloidal suspension (such as a stable suspension of nanoparticles). In some embodiments, the carrier protein is in an amount that reduces the sedimentation rate of the taxane in an aqueous medium. For particle-containing compositions, the amount of the carrier protein also depends on the size and density of nanoparticles of the taxane.

[0169] A taxane is "stabilized" in an aqueous suspension if it remains suspended in an aqueous medium (such as without visible precipitation or sedimentation) for an extended period of time, such as for at least about any of 0.1, 0.2, 0.25, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 24, 36, 48, 60, or 72 hours. The suspension is generally, but not necessarily, suitable for administration to an individual (such as human). Stability of the suspension is generally (but not necessarily) evaluated at a storage temperature (such as room temperature (such as 20-25 °C) or refrigerated conditions (such as 4 °C)). For example, a suspension is stable at a storage temperature if it exhibits no flocculation or particle agglomeration visible to the naked eye or when viewed under the optical microscope at 1000 times, at about fifteen minutes after preparation of the suspension. Stability can also be evaluated under accelerated testing conditions, such as at a temperature that is higher than about 40 °C.

[0170] In some embodiments, the carrier protein is present in an amount that is sufficient to stabilize the taxane in an aqueous suspension at a certain concentration. For example, the concentration of the taxane in the composition is about 0.1 to about 100 mg/ml, including for example any of about 0.1 to about 50 mg/ml, about 0.1 to about 20 mg/ml, about 1 to about 10 mg/ml, about 2 mg/ml to about 8 mg/ml, about 4 to about 6 mg/ml, about 5 mg /ml. In some embodiments, the concentration of the taxane is at least about any of 1.3 mg/ml, 1.5 mg/ml, 2 mg/ml, 3 mg/ml, 4 mg/ml, 5 mg/ml, 6 mg/ml, 7 mg/ml, 8 mg/ml, 9 mg/ml, 10 mg/ml, 15 mg/ml, 20 mg/ml, 25 mg/ml, 30 mg/ml, 40 mg/ml, and 50 mg/ml. In some embodiments, the carrier protein is present in an amount that avoids use of surfactants (such as Cremophor), so that the composition is free or substantially free of surfactant (such as Cremophor).

[0171] In some embodiments, the composition, in liquid form, comprises from about 0.1% to about 50% (w/v) (e.g. about 0.5% (w/v), about 5% (w/v), about 10% (w/v), about 15% (w/v), about 20% (w/v), about 30% (w/v), about 40% (w/v), or about 50% (w/v)) of carrier protein. In some embodiments, the composition, in liquid form, comprises about 0.5% to about 5% (w/v) of carrier protein.

[0172] In some embodiments, the weight ratio of carrier protein, e.g., albumin, to the taxane in the nanoparticle composition is such that a sufficient amount of taxane binds to, or is transported by, the cell. While the weight ratio of carrier protein to taxane will have to be optimized for different carrier protein and taxane combinations, generally the weight ratio of carrier protein, e.g., albumin, to taxane (w/w) is about 0.01:1 to about 100:1, about 0.02:1 to about 50:1, about 0.05:1 to about 20:1, about 0.1:1 to about 20:1, about 1:1 to about 18:1, about 2:1 to about 15:1, about 3:1 to about 12:1, about 4:1 to about 10:1, about 5:1 to about 9:1, or about 9:1. In some embodiments, the carrier protein to taxane weight ratio is about any of 18:1 or less, 15:1 or less, 14:1 or less, 13:1 or less, 12:1 or less, 11:1 or less, 10:1 or less, 9:1 or less, 8:1 or less, 7:1 or less, 6:1 or less, 5:1 or less, 4:1 or less, and 3:1 or less.

[0173] In some embodiments, the carrier protein allows the composition to be administered to an individual (such as human) without significant side effects. In some embodiments, the carrier protein (such as albumin) is in an amount that is effective to reduce one or more side effects of administration of the taxane to a human. The term "reducing one or more side effects of administration of the taxane" refers to reduction, alleviation, elimination, or avoidance of one or more undesirable effects caused by the taxane, as well as side effects caused by delivery vehicles (such as solvents that render the taxanes suitable for injection) used to deliver the taxane. Such side effects include, for example, myelosuppression, neurotoxicity, hypersensitivity, inflammation, venous irritation, phlebitis, pain, skin irritation, peripheral neuropathy, neutropenic fever, anaphylactic reaction, venous thrombosis, extravasation, and combinations thereof. These side effects, however, are merely exemplary and other side effects, or combination of side effects, associated with taxanes can be reduced.

[0174] In some embodiments, the composition comprises Abraxane™. Abraxane™ is a formulation of paclitaxel stabilized by human albumin USP, which can be dispersed in directly injectable physiological solution. When dispersed in a suitable aqueous medium such as 0.9% sodium chloride injection or 5% dextrose injection, Abraxane™ forms a stable colloidal suspension of paclitaxel. The mean particle size of the nanoparticles in the colloidal suspension is about 130 nanometers. Since HSA is freely soluble in water, Abraxane™ can be reconstituted in a wide range of concentrations ranging from dilute (0.1 mg/ml paclitaxel) to concentrated (20 mg/ml paclitaxel), including for example about 2 mg/ml to about 8 mg/ml, about 5 mg/ml.

[0175] Methods of making nanoparticle compositions are known in the art. For example, nanoparticles containing taxanes (such as paclitaxel) and carrier protein (such as albumin) can be prepared under conditions of high shear forces (e.g., sonication, high pressure homogenization, or the like). These methods are disclosed in, for example, U.S. Pat. Nos. 5,916,596; 6,506,405; and 6,537,579 and also in U.S. Pat. Pub. No. 2005/0004002A1.

[0176] Briefly, the taxane (such as docetaxel) is dissolved in an organic solvent, and the solution can be added to a human serum albumin solution. The mixture is subjected to high pressure homogenization. The organic solvent can then be removed by evaporation. The dispersion obtained can be further lyophilized. Suitable organic solvent include, for example, ketones, esters, ethers, chlorinated solvents, and other solvents known in the art. For example, the organic solvent can be methylene chloride and chloroform/ethanol (for example with a ratio of 1:9, 1:8, 1:7, 1:6, 1:5, 1:4, 1:3, 1:2, 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, or 9:a).

Other components in the nanoparticle compositions

[0177] The nanoparticles described herein can be present in a composition that include other agents, excipients, or stabilizers. For example, to increase stability by increasing the negative zeta potential of nanoparticles, certain negatively charged components may be added. Such negatively charged components include, but are not limited to bile salts of bile acids consisting of glycocholic acid, cholic acid, chenodeoxycholic acid, taurocholic acid, glycochenodeoxycholic acid, taurochenodeoxycholic acid, litocholic acid, ursodeoxycholic acid, dehydrocholic acid and others; phospholipids including lecithin (egg yolk) based phospholipids which include the following phosphatidylcholines: palmitoyloleoylphosphatidylcholine, palmitoyllinoleoylphosphatidylcholine , stearoyllinoleoylphosphatidylcholine stearoyloleoylphosphatidylcholine, stearoylarachidoylphosphatidylcholine, and dipalmitoylphosphatidylcholine. Other phospholipids including L-α-dimyristoylphosphatidylcholine (DMPC), dioleoylphosphatidylcholine (DOPC), distearyolphosphatidylcholine (DSPC), hydrogenated soy phosphatidylcholine (HSPC), and other related compounds. Negatively charged surfactants or emulsifiers are also suitable as additives, e.g., sodium cholesteryl sulfate and the like.

[0178] In some embodiments, the composition is suitable for administration to a human. In some embodiments, the composition is suitable for administration to a mammal

such as, in the veterinary context, domestic pets and agricultural animals. There are a wide variety of suitable formulations of the nanoparticle composition (see, e.g., U.S. Pat. Nos. 5,916,596 and 6,096,331). The following formulations and methods are merely exemplary and are in no way limiting. Formulations suitable for oral administration can consist of (a) liquid solutions, such as an effective amount of the compound dissolved in diluents, such as water, saline, or orange juice, (b) capsules, sachets or tablets, each containing a predetermined amount of the active ingredient, as solids or granules, (c) suspensions in an appropriate liquid, and (d) suitable emulsions. Tablet forms can include one or more of lactose, mannitol, corn starch, potato starch, microcrystalline cellulose, acacia, gelatin, colloidal silicon dioxide, croscarmellose sodium, talc, magnesium stearate, stearic acid, and other excipients, colorants, diluents, buffering agents, moistening agents, preservatives, flavoring agents, and pharmacologically compatible excipients. Lozenge forms can comprise the active ingredient in a flavor, usually sucrose and acacia or tragacanth, as well as pastilles comprising the active ingredient in an inert base, such as gelatin and glycerin, or sucrose and acacia, emulsions, gels, and the like containing, in addition to the active ingredient, such excipients as are known in the art.

[0179] Examples of suitable carriers, excipients, and diluents include, but are not limited to, lactose, dextrose, sucrose, sorbitol, mannitol, starches, gum acacia, calcium phosphate, alginates, tragacanth, gelatin, calcium silicate, microcrystalline cellulose, polyvinylpyrrolidone, cellulose, water, saline solution, syrup, methylcellulose, methyl- and propylhydroxybenzoates, talc, magnesium stearate, and mineral oil. The formulations can additionally include lubricating agents, wetting agents, emulsifying and suspending agents, preserving agents, sweetening agents or flavoring agents.

[0180] Formulations suitable for parenteral administration include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain anti-oxidants, buffers, bacteriostats, and solutes that render the formulation compatible with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. The formulations can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials, and can be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid excipient, for example, water, for injections, immediately prior to use. Extemporaneous injection solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described. Injectable formulations are preferred.

[0181] In some embodiments, the composition is formulated to have a pH range of about 4.5 to about 9.0, including for example pH ranges of any of about 5.0 to about 8.0, about 6.5 to about 7.5, and about 6.5 to about 7.0. In some embodiments, the pH of the composition is formulated to no less than about 6, including for example no less than about any of 6.5, 7, or 8 (such as about 8). The composition can also be made to be isotonic with blood by the addition of a suitable tonicity modifier, such as glycerol.

Kits

[0182] The invention also provides kits for use in the instant methods. Kits of the invention include one or more containers comprising taxane-containing nanoparticle compositions (or unit dosage forms and/or articles of manufacture) and/or a chemotherapeutic agent, and in some embodiments, further comprise instructions for use in accordance with any of the methods described herein. The kit may further comprise a description of selection an individual suitable or treatment. Instructions supplied in the kits of the invention are typically written instructions on a label or package insert (e.g., a paper sheet included in the kit), but machine-readable instructions (e.g., instructions carried on a magnetic or optical storage disk) are also acceptable.

[0183] In some embodiments, the kit comprises a) a composition comprising nanoparticles comprising a taxane and a carrier protein (such as albumin), b) an effective amount of at least one other chemotherapeutic agent, and c) instructions for administering the nanoparticles and the chemotherapeutic agents simultaneously and/or sequentially, for treatment of a proliferative disease (such as cancer). In some embodiments, the taxane is any of paclitaxel, docetaxel, and ortataxel. In some embodiments, the kit comprises nanoparticles comprising a) a composition comprising nanoparticles comprising paclitaxel and an albumin (such as Abraxane^{TM}), b) an effective amount of at least one other chemotherapeutic agent, and c) instructions for administering the nanoparticles and the chemotherapeutic agents simultaneously and/or sequentially, for the effective treatment of a proliferative disease (such as cancer).

[0184] In some embodiments, the kit comprises a) a composition comprising nanoparticles comprising a taxane and a carrier protein (such as albumin), b) a composition comprising nanoparticles comprising at least one other chemotherapeutic agent and a carrier protein (such as albumin), and c) instructions for administering the nanoparticle compositions simultaneously and/or sequentially, for treatment of a proliferative disease (such as cancer). In some embodiments, the kit comprises nanoparticles comprising a) a

composition comprising nanoparticles comprising paclitaxel and an albumin (such as Abraxane™), b) a composition comprising nanoparticles comprising at least one other chemotherapeutic agent and a carrier protein (such as albumin), and c) instructions for administering the nanoparticle compositions simultaneously and/or sequentially, for the effective treatment of a proliferative disease (such as cancer).

[0185] The nanoparticles and the chemotherapeutic agents can be present in separate containers or in a single container. It is understood that the kit may comprise one distinct composition or two or more compositions wherein one composition comprises nanoparticles and one composition comprises a chemotherapeutic agent.

[0186] The kits of the invention are in suitable packaging. Suitable packaging include, but is not limited to, vials, bottles, jars, flexible packaging (e.g., seled Mylar or plastic bags), and the like. Kits may optionally provide additional components such as buffers and interpretative information.

[0187] The instructions relating to the use of the nanoparticle compositions generally include information as to dosage, dosing schedule, and route of administration for the intended treatment. The containers may be unit doses, bulk packages (e.g., multi-dose packages) or sub-unit doses. For example, kits may be provided that contain sufficient dosages of the taxane (such as taxane) as disclosed herein to provide effective treatment of an individual for an extended period, such as any of a week, 2 weeks, 3 weeks, 4 weeks, 6 weeks, 8 weeks, 3 months, 4 months, 5 months, 7 months, 8 months, 9 months, or more. Kits may also include multiple unit doses of the taxane and pharmaceutical compositions and instructions for use and packaged in quantities sufficient for storage and use in pharmacies, for example, hospital pharmacies and compounding pharmacies.

[0188] Those skilled in the art will recognize that several variations are possible within the scope and spirit of this invention. The invention will now be described in greater detail by reference to the following non-limiting examples. The following examples further illustrate the invention but, of course, should not be construed as in any way limiting its scope.

EXAMPLES

Example 1. Improved response and reduced toxicities for Abraxane™ compared to Taxol® in a Phase III study of Abraxane™ given every three weeks.

[0189] Significantly reduced incidence of neutropenia and hypersensitivity, absence of requirement of steroid premedication, shorter duration of neuropathy, shorter infusion time and higher dose.

[0190] ABI-007 (Abraxane™), the first biologically interactive albumin-bound paclitaxel in a nanoparticle form, free of any solvent, was compared with Cremophor®-based paclitaxel (Taxol®) in individuals with metastatic breast cancer (MBC). This phase III study was performed to confirm the preclinical studies demonstrating superior efficacy and reduced toxicity of ABI-007 when compared with Taxol®. Individuals were randomly assigned to 3-week cycles of either ABI-007 260 mg/m^2 (iv) over 30 minutes without premedication (n = 229) or Taxol® 175 mg/m^2 IV over 3 hours with premedication (n = 225). ABI-007 demonstrated significantly higher response rates compared with Taxol® (33% vs. 19%; p = 0.001) and significantly longer time to tumor progression (23.0 vs. 16.9 weeks; HR = 0.75; p = 0.006). There was a trend for longer overall survival in individuals who received ABI-007 (65.0 vs. 55.7 weeks; p = 0.374). In an unplanned analysis, ABI-007 improved survival in individuals receiving treatment as second- or greater-line therapy (56.4 vs. 46.7 weeks; HR = 0.73; p = 0.024). The incidence of grade 4 neutropenia was significantly lower in the ABI-007 group (9% vs. 22%; p < 0.001) despite a 49% higher paclitaxel dose. Grade 3 sensory neuropathy was more common in the ABI-007 group than in the Taxol® group (10% vs. 2%; p < 0.001) but was easily managed and improved more rapidly (median, 22 days) than for Taxol® (median 73 days). No severe (grade 3 or 4) treatment-related hypersensitivity reactions occurred in any of the individuals in the ABI-007 group despite the absence of premedication and shorter administration time. In contrast, grade 3 hypersensitivity reactions occurred in the Taxol® group despite standard premedication (chest pain: 2 individuals; allergic reaction: 3 individuals). Per protocol, corticosteroids and antihistamines were not administered routinely to individuals in the ABI-007 group; however, premedication was administered for emesis, myalgia/arthralgia, or anorexia in 18 individuals (8%) in the ABI-007 group in 2% of the treatment cycles, whereas 224 individuals (>99%) in the Taxol® group received premedication at 95% of the cycles. The only clinical chemistry value that was notably different between the 2 treatment arms was higher serum glucose levels in the Taxol®-treated individuals, who also had a higher incidence of hyperglycemia reported as an AE (adverse effects) (15 [7%] vs. 3 [1%]; p = 0.003). Overall, ABI-007 demonstrated greater efficacy and a favorable safety profile compared with Taxol® in this individual population. The improved therapeutic index and elimination of the steroid premedication required for

solvent-based taxanes make this nanoparticle albumin-bound paclitaxel an important advance in the treatment of MBC.

Example 2. Weekly Abraxane™ in Taxane-Refractory Metastatic Breast Cancer Individuals

[0191] A recent Phase II clinical study showed that weekly administration of Abraxane™ (nanoparticle albumin-bound paclitaxel) at a dose of 125 mg/m^2 resulted in long-term disease control in individuals with metastatic breast cancer whose disease had progressed while being treated with Taxol® or Taxotere® (that is, individuals who are taxane-refractory).

[0192] Abraxane™ is believed to represent the first biologically interactive composition that exploits the receptor-mediated (gp60) pathway found to be integral to achieving high intracellular tumor concentrations of the active ingredient - paclitaxel. The Phase II study included 75 individuals with taxane-refractory metastatic breast cancer. Abraxane™ was administered weekly via a 30-minute infusion at 125 mg/m^2 without steroid/antihistamine premedication or G-CSF prophylaxis. Individuals received three weekly doses followed by one week of rest, repeated every 28 days. Unlike Taxol® or Taxotere®, which contain detergents that may inhibit tumor uptake, the mechanism of action of the albumin-bound nanoparticle paclitaxel may result in improved outcomes, especially in this difficult-to-treat individual population.

[0193] Specifically, the data showed that despite this high weekly dose of 125 mg/m^2 in this highly pre-treated and prior taxane-exposed individual population, only 3 of 75 individuals (4%) had to discontinue Abraxane™ due to peripheral neuropathy. Furthermore, of those who experienced Grade 3 peripheral neuropathy, 80% were typically able to resume treatment after a delay of only 1 or 2 weeks and continued to receive Abraxane™ at a reduced dose for an average of 4 additional months. This rapid improvement was consistent with our observation from the Phase III trial - that the peripheral neuropathy induced by paclitaxel alone (i.e., without Cremophor®) improves rapidly as compared to that induced by Taxol®. These Abraxane™ clinical trial experiences provide the first clinical opportunity to evaluate the effects of the chemotherapeutic agent itself, paclitaxel, from the effects from those of solvents. Based upon both the Phase II and III experience, the data now suggest that the peripheral neuropathy from Abraxane™ is not comparable to the peripheral neuropathy from Taxol® or Taxotere® with respect to duration and impact on the individual.

[0194] With regard to the clinical experience of peripheral neuropathy following Taxol® or Taxotere®, Abraxis Oncology recently completed a survey of 200 oncologists who were asked how long they thought the peripheral neuropathy induced by Taxol® took to improve and/or resolve: 25% reported "7-12 months" and another 23% reported "never resolved"; for Taxotere®, the respective percentages were 29% and 7%. These data are consistent with the statements in the Taxotere® and Taxol® package inserts.

[0195] Analysis of the Phase II data demonstrates Abraxane™ to be active in this poor-prognosis individual population (87% visceral (lung and liver) disease, 69% >3 metastatic sites, 88% tumor growth while on taxanes), of taxane-refractory individuals with metastatic breast cancer. Observations included a 44% disease control in Taxotere®-refractory individuals and 39% disease control in Taxol®-refractory individuals. Of those individuals whose disease progressed while on Taxotere® alone in the metastatic setting (n=27) a 19% response rate was noted after receiving weekly Abraxane™. Of those individuals whose disease progressed while on Taxol® alone in the metastatic setting (n=23) a 13% response rate was noted after receiving weekly Abraxane™.

[0196] Abraxane™ was found to be well tolerated when administered weekly over 30 minutes without steroids or G-CSF prophylaxis: Grade 4 neutropenia = 3% (without G-CSF); Grade 4 anemia = 1%; no severe hypersensitivity reactions (despite absence of premedication). In this heavily pretreated individual population, 75% of individuals were treated at the full high dose of 125 mg/m^2 weekly Abraxane™, with no dose reductions due to toxicities/adverse events. Of the individuals who developed grade 3 sensory neuropathy, 77% were able to restart Abraxane™ at a reduced dose (75-100 mg/m^2) and received a mean of 12.2 (range, 1-28) additional doses of Abraxane™. It was remarkable to note that of these individuals who resumed Abraxane™, 80% (8 of 10) were able to restart the drug within 14 days after improvement of neuropathy to Grade 1 or 2. These results support the observations in the pivotal Phase III trial of 260 mg/m^2 Abraxane™ administered every 3 weeks, in which rapid improvement of neuropathy (median of 22 days) was also noted. Taken together these two clinical trials suggest when paclitaxel is given alone, the neuropathy which occurs appears to be short-lived and is easily managed.

[0197] Abraxane™ utilizes the gp60 receptor based pathway on the microvessel endothelial cells to transport the albumin-paclitaxel complex out of the blood vessel and into the tumor interstitium, and it has been shown that Taxol® was not transported by this mechanism. Furthermore, an albumin-binding protein, SPARC, was over-expressed in breast tumors and may play a role in the increased intra-tumoral accumulation of

Abraxane™. The proposed mechanism suggested that once in the tumor interstitium, the albumin-paclitaxel complex would bind to SPARC that was present on the tumor cell surface and be rapidly internalized into the tumor cell by a non-lysosomal mechanism.

[0198] In addition, the surfactants/solvents commonly used in current taxane formulations such as Cremophor®, Tween® 80 and TPGS, strongly inhibit the binding of paclitaxel to albumin, thereby limiting transendothelial transport. Additional data presented showed a statistically improved efficacy of Abraxane™ over Taxotere® in the MX-1 mammary breast carcinoma xenograft at equal dose.

[0199] In conclusion, 75% of individuals were treated at full high dose with no dose reductions. Data indicate rapid improvement of peripheral neuropathy when nanoparticle albumin-bound paclitaxel is administered alone, without the solvent Cremophor®. Additional data provide increased evidence that mechanism of action may play important role in enhancing individual outcomes.

Example 3. Abraxane™ (ABI-007) acts synergistically with targeted antiangiogenic pro-apoptotic peptides (HKP) in MDA-MB-435 human tumor xenografts.

[0200] The antiangiogenic activity of small synthetic pro-apoptotic peptides composed of two functional domains, one targeting the CD13 receptors (aminopeptidase N) on tumor microvessels and the other disrupting the mitochondrial membrane following internalization have previously been reported. See Nat Med. 1999 Sep; 5(9):1032-8. A second generation dimeric peptide, CNGRC-GG-d(KLAKLAK)$_2$, named HKP (Hunter Killer Peptide) was found to have improved antitumor activity. Since anti-angiogenic agents such as Avastin® exhibit synergism in combination with cytotoxic agents such as 5-fluorouracil, we evaluated the combination of the antiangiogenic HKP with Abraxane™ (ABI-007), an albumin nanoparticle paclitaxel that is transported by the gp60 receptor in vascular endothelium (Desai, SABCS 2003), in MDA-MB-435 human breast tumor xenografts.

[0201] Methods: MDA-MB-435 human tumor xenografts were established at an average tumor volume of 100 mm^3, mice were randomized into groups of 12-13 animals and treated with HKP, Abraxane™, or HKP and Abraxane™. HKP was delivered i.v. (250 ug), once a week, for 16 weeks. Abraxane™ was administered i.v., daily for 5 days at 10 mg/kg/day only for the first week of treatment. The Abraxane™ dose used was substantially below its MTD (30 mg/kg/day, qd x 5) to prevent the tumor from complete regression so effect of HKP could be noted.

[0202] Results: At nineteen weeks of treatment, tumor volume was significantly decreased between control group (10,298 $mm^3 \pm 2,570$) and HKP (4,372 $mm^3 \pm 2,470$; $p < 0.05$ vs control) or ABI-007 (3,909 $mm^3 \pm 506$; $p < 0.01$ vs control). The combination of ABI-007 and HKP significantly reduced the tumor volume over either monotherapy (411 $mm^3 \pm 386$; $p < 0.01$ vs. Abraxane™ monotherapy or HKP monotherapy). The treatments were well tolerated.

[0203] Conclusion: The combination of Abraxane™ (ABI-007), a nanoparticle albumin-bound paclitaxel, with the vascular targeting anti-angiogenic dimeric peptide HKP (CNGRC-GG-d(KLAKLAK)$_2$) against the MDA-MB-435 xenograft breast tumor showed a significant reduction in tumor volume compared to monotherapy of either agent alone. Our results suggest that the combination of Abraxane™ with antiangiogenic agents such as HKPs or perhaps Avastin® may be beneficial.

Example 4. Metronomic ABI-007 Therapy: Antiangiogenic and Antitumor Activity of a Nanoparticle Albumin-bound Paclitaxel

Example 4a

[0204] Methods: The antiangiogenic activity of ABI-007 was assessed by the rat aortic ring, human umbilical vein endothelial cell (HUVEC) proliferation and tube formation assays. Optimal dose of ABI-007 for metronomic therapy was determined by measuring the levels of circulating endothelial progenitors (CEPs) in peripheral blood of Balb/c non-tumor bearing mice (n=5/group; dosing: 1–30 mg/kg, i.p, qd x 7) with flow cytometry (Shaked et al., *Cancer Cell*, 7:101-111 (2005)). Subsequently, the antitumor effects of metronomic (qd; i.p.) and MTD (qd x 5, 1 cycle; i.v.) ABI-007 and Taxol® were evaluated and compared in SCID mice bearing human MDA-MD-231 breast and PC3 prostate cancer xenografts.

[0205] Results: ABI-007 at 5 nM significantly ($p < 0.05$) inhibited rat aortic microvessel outgrowth, human endothelial cell proliferation and tube formation by 53%, 24%, and 75%, respectively. The optimal dose of ABI-007 for metronomic therapy was observed to be 6-10 mg/kg based on CEP measurements. Metronomic ABI-007 (6 mg/kg) but not Taxol® (1.3 mg/kg) significantly ($p < 0.05$) suppressed tumor growth in both xenograft models. Neither ABI-007 nor Taxol® administered metronomically induced any weight loss. Although MTD ABI-007 (30 mg/kg) inhibited tumor growth more effectively than MTD Taxol® (13 mg/kg), significant weight loss was noted with the former.

Interestingly, the antitumor effect of metronomic ABI-007 approximated that of MTD Taxol®.

[0206] Conclusion: ABI-007 exhibits potent antiangiogenic and antitumor activity when used in a metronomic regime.

Example 4b

[0207] Rat Aortic Ring Assay. Twelve-well tissue culture plates were coated with Matrigel (Collaborative Biomedical Products, Bedford, MA) and allowed to gel for 30 min at 37°C and 5% CO_2. Thoracic aortas were excised from 8- to 10-week-old male Sprague-Dawley rats, cut into 1-mm-long cross-sections, placed on Matrigel-coated wells and covered with an additional Matrigel. After the second layer of Matrigel had set, the rings were covered with EGM-II and incubated overnight at 37°C and 5% CO_2. EGM-II consists of endothelial cell basal medium (EBM-II; Cambrex, Walkersville, MD) plus endothelial cell growth factors provided as the EGM-II Bulletkit (Cambrex). The culture medium was subsequently changed to EBM-II supplemented with 2% FBS, 0.25 μg/ml amphotericin B and 10 μg/ml gentamycin. Aortic rings were treated with EBM-II containing the vehicle (0.9% saline/albumin), carboxyamidotriazole (CAI; 12 μg/ml), or ABI-007 (0.05-10 nM paclitaxel) for 4 days and photographed on the fifth day. CAI, a known anti-angiogenic agent, was used at a higher than clinically achievable concentration as a positive control. Experiments were repeated four times using aortas from four different rats. The area of angiogenic sprouting, reported in square pixels, was quantified using Adobe Photoshop 6.0.

[0208] As shown in Figure 1A, ABI-007 significantly inhibited rat aortic microvessel outgrowth in a concentration-dependent manner relative to the vehicle control, reaching statistical significance ($p < 0.05$) at 5 nM (53% inhibition) and 10 nM (68% inhibition). The amount of albumin present at each concentration of ABI-007 alone did not inhibit angiogenesis.

[0209] Endothelial Cell Proliferation Assay. Human umbilical vein endothelial cells (HUVEC; Cambrex) were maintained in EGM-II at 37°C and 5% CO2. HUVECs were seeded onto 12-well plates at a density of 30,000 cells/well and allowed to attach overnight. The culture medium was then aspirated, and fresh culture medium containing either the vehicle (0.9% saline/albumin), or ABI-007 (0.05-10 nM paclitaxel) was added to each well. After 48 h, cells were trypsinized and counted with a Coulter Z1 counter (Coulter Corp., Hialeah, FL). All experiments were repeated three times.

[0210] As shown in Figure 1B, human endothelial cell proliferation was significantly inhibited by ABI-007 at 5 nM and 10 nM by 36% and 41%, respectively.

[0211] Endothelial Cell Tube Formation Assay. Eight-well slide chambers were coated with Matrigel and allowed to gel at 37°C and 5% CO_2 for 30 min. HUVECs were then seeded at 30,000 cells/well in EGM-II containing either the vehicle (0.9% saline/albumin) or ABI-007 (0.05-10 nM paclitaxel) and incubated at 37°C and 5% CO_2 for 16 h. After incubation, slides were washed in PBS, fixed in 100% methanol for 10 s, and stained with DiffQuick solution II (Dade Behring Inc., Newark, DE) for 2 min. To analyze tube formation, each well was digitally photographed using a 2.5x objective. A threshold level was set to mask the stained tubes. The corresponding area was measured as the number of pixels using MetaMorph software (Universal Imaging, Downingtown, PA). Experiments were repeated three times.

[0212] As shown in Figure 1C, ABI-007 blocked tube formation by 75% at both 5 nM and 10 nM.

[0213] Determination of the In Vivo Optimal Biologic Dose of ABI-007 by Measuring Circulating Endothelial Cells (CECs) and Circulating Endothelial Progenitors (CEPs). Six- to eight-week-old female Balb/cJ mice were randomized into the following eight groups (n=5 each): untreated, treated with i.p. bolus injections of either the drug vehicle (0.9% saline/ albumin), or ABI-007 at 1, 3, 6, 10, 15 or 30 mg/kg paclitaxel daily for 7 days. At the end of the treatment period, blood samples were drawn by cardiac puncture and collected in EDTA-containing vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ). CECs and CEPs were enumerated using four-color flow cytometry. Monoclonal antibodies specific for CD45 were used to exclude CD45+ hematopoietic cells. CECs and their CEP subset were depicted using the murine endothelial markers fetal liver kinase 1/VEGF receptor 2 (flk-1/VEGFR2), CD13, and CD117 (BD Pharmingen, San Diego, CA). Nuclear staining (Procount; BD Biosciences, San Jose, CA) was performed to exclude the possibility of platelets or cellular debris interfering with the accuracy of CEC and CEP enumeration. After red cell lysis, cell suspensions were evaluated by a FACSCalibur (BD Biosciences) using analysis gates designed to exclude dead cells, platelets, and debris. At least 100,000 events/sample were obtained in order to analyze the percentage of CECs and CEPs. The absolute number of CECs and CEPs was then calculated as the percentage of the events collected in the CEC and CEP enumeration gates multiplied by the total white cell count. Percentages of stained cells were determined and compared to the appropriate negative controls. Positive staining was defined as being

greater than non-specific background staining. 7-aminoactinomycin D (7AAD) was used to enumerate viable versus apoptotic and dead cells.

[0214] Figure 2 shows that ABI-007 administered i.p. daily for 7 days at 3, 10-30 mg/kg significantly decreased CEP levels in non-tumor bearing Balb/cJ mice. However, ABI-007 at 10-30 mg/kg was associated with a significant reduction of white blood cell count indicative of toxicity. Although the reduction of CEP levels by ABI-007 at 6 mg/kg did not reach statistical significance, decrease in white blood cell count was not evident. Therefore it was concluded that the in vivo optimal biologic dose for metronomic ABI-007 was between 3-10 mg/kg. In one study, metronomic Taxol® at 1.3, 3, 6, or 13 mg/kg given i.p. daily for 7 days did not significantly reduce viable CEP levels, whereas metronomic Taxol® at 30 mg/kg or higher resulted in severe toxicity and eventually mortality in mice. It was previously reported that the i.p. administration of Taxol® at doses commonly used in the clinic resulted in entrapment of paclitaxel in Cremophor® EL micelles in the peritoneal cavity and consequently, insignificant plasma paclitaxel concentration (Gelderblom et al., *Clin. Cancer Res.* 8:1237-41 (2002)). This would explain why doses of metronomic Taxol® (1.3, 3, 6, and 13 mg/kg) that did not cause death failed to change viable CEP levels. In this case, the i.p. administration of metronomic Taxol® at 1.3 mg/kg would not be any different from that at 13 mg/kg. Therefore the lower dose, 1.3 mg/kg, was selected to minimize the amount of Cremophor® EL per paclitaxel administration for subsequent experiments.

[0215] Antitumor effects of metronomic and MTD ABI-007 compared with metronomic and MTD Taxol®. Human prostate cancer cell line PC3 and human breast cancer cell line MDA-MD-231 were obtained from the American Type Culture Collection (Manassas, VA). PC3 cells (5×10^6) were injected s.c. into 6- to 8-week-old male SCID mice, whereas MDA-MB-231 cells (2×10^6) were implanted orthotopically into the mammary fat pad of female SCID mice. When the primary tumor volume reached approximately 150-200 mm^3, animals were randomized into eight groups (n=5-10/group). Each group was treated with either 0.9% saline/albumin vehicle control, Cremophor® EL vehicle control, metronomic Taxol® (1.3 mg/kg, i.p., qd), metronomic ABI-007 (3, 6, or 10 mg/kg paclitaxel, i.p., qd), MTD Taxol® (13 mg/kg, i.p., qd x 5, 1 cycle), or MTD ABI-007 (30 mg/kg paclitaxel, i.v., qd x 5, 1 cycle). Perpendicular tumor diameters were measured with a caliper once a week and their volumes were calculated. At the end of the treatment period, blood samples were drawn by cardiac puncture from mice in all groups. CECs and CEPs were enumerated as described herein.

[0216] Metronomic ABI-007 (3, 6 and 10 mg/kg) but not Taxol® (1.3 mg/kg) administered i.p. daily for 4 weeks significantly ($p < 0.05$) inhibited growth of both MDA-MB-231 and PC3 tumors (Fig. 3A and Fig. 3B). Neither ABI-007 nor Taxol® administered metronomically induced any weight loss (Fig. 3C and Fig. 3D). Although MTD ABI-007 (30 mg/kg) inhibited tumor growth more effectively than MTD Taxol® (13 mg/kg), significant weight loss was noted with the former, indicating toxicity. In addition, two out of five mice treated with MTD ABI-007 displayed signs of paralysis in one limb 6 days after the last dose of drug. The paralysis was transient and resolved within 24-48 hours. Interestingly, the antitumor effect of metronomic ABI-007 at 6 mg/kg approximated that of MTD Taxol® in the MDA-MB-231 xenograft model (Fig. 3A). Increasing the dose of metronomic ABI-007 to 10 mg/kg did not seem to confer more pronounced tumor growth inhibition. In contrast, metronomic ABI-007 elicited greater antitumor response at 10 mg/kg than at 3 and 6 mg/kg in the PC3 xenografts (Fig. 3B).

[0217] Metronomic ABI-007 significantly decreased the levels of viable CEPs in a dose-dependent manner in MDA-MB-231 tumor-bearing mice (Fig. 4A). Viable CEP levels also exhibited a dose-dependent reduction in response to metronomic ABI-007 in PC3 tumor-bearing mice, but reached statistical significance only at 10 mg/kg (Fig. 4B). The levels of CEPs were not altered by metronomic Taxol® in both xenograft models (Fig. 4A and 4B).

[0218] Effects of metronomic and MTD ABI-007 and metronomic and MTD Taxol® on intratumoral microvessel density were studied. Five-um thick sections obtained from frozen MDA-MB-231 and PC3 tumors were stained with H&E for histological examination by standard methods known to one skilled in the art. For detection of microvessels, sections were stained with a rat anti-mouse CD31/PECAM-1 antibody (1:1000, BD Pharmingen) followed by a Texas Red-conjugated goat anti-rat secondary antibody (1:200, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). A single microvessel was defined as a discrete cluster or single cell stained positive for CD31/PECAM-1d, and the presence of a lumen was not required for scoring as a microvessel. The MVD for each tumor was expressed as the average count of the three most densely stained fields identified with a 20x objective on a Zeiss AxioVision 3.0 fluorescence microscopic imaging system. Four to five different tumors per each vehicle control or treatment group were analyzed.

[0219] In MDA-MB-231 tumors, metronomic ABI-007 at 6 and 10 mg/kg as well as MTD ABI-007 seemed to reduce microvessel density (MVD) slightly although statistical

significance was not reached (Fig. 5A). In PC3 tumors, metronomic ABI-007 at 3 and 10 mg/kg appeared to decrease MVD but without reaching statistical significance (Fig. 5A). Interestingly, a significant correlation existed between MVD and the level of viable CEPs in the MDA-MB-231 (Fig. 5B; r=0.76, P-0.04) but not in the PC3 (Fig. 5C; r=-0.071, P-0.88) model.

[0220] In vivo angiogenesis evaluation were carried out. A Matrigel plug perfusion assay was performed with minor modifications to methods known by one skilled in the art. Briefly, 0.5 ml Matrigel supplemented with 500 ng/ml of basic fibroblast growth factor (bFGF; R&D Systems Inc., Minneapolis, MN) was injected s.c. on day 0 into the flanks of 10-week-old female Balb/cJ mice. On day 3, animals were randomly assigned to eight groups (n = 5 each). Each group was treated with either 0.9% saline/albumin vehicle control, Cremophor® EL vehicle control, metronomic Taxol® (1.3 mg/kg, i.p., qd), metronomic ABI-007 (3, 6, or 10 mg/kg paclitaxel, i.p., qd), MTD Taxol® (13 mg/kg, i.v., qd x 5), or MTD ABI-007 (30 mg/kg paclitaxel, i.v, qd x 5). As a negative control, five additional female Balb/cJ mice of similar age were injected with Matrigel alone. On day 10, all animals were injected i.v. with 0.2 ml of 25 mg/ml FITC-dextran (Sigma, St. Louis, MO). Plasma samples were subsequently collected. Matrigel plugs were removed, incubated with Dispase (Collaborative Biomedical Products, Bedford, MA) overnight at 37°C, and then homogenized. Fluorescence readings were obtained using a FL600 fluorescence plate reader (Biotech Instruments, Winooski, VT). Angiogenic response was expressed as the ratio of Matrigel plug fluorescence to plasma fluorescence.

[0221] Metronomic ABI-007 at 6 and 10 mg/kg appeared to decrease angiogenesis although the inhibition did not reach statistical significance (Fig. 6). Angiogenesis seemed to be unaltered by metronomic ABI-007 at 3 mg/kg, MTD ABI-007, MTD and metronomic Taxol® relative to the respective vehicle controls (Fig. 6). These observations were similar to the intratumoral MVD results described herein.

Example 5. Nab-5109, A Nanoparticle albumin-bound IDN5109 (nab-5109) Shows Improved Efficacy and Lower Toxicity over the Tween® formulation (Tween®-5109, Ortataxel)

[0222] Methods: Nanoparticle nab-5109 was prepared using *nab* technology and characterized by laser light scattering. Nab-5109 and Tween-5109 were tested against Pgp+ DLD-1 (known to be resistant against paclitaxel and docetaxel - Vredenburg et al, *JNCI* 93: 1234-1245, 2001) human colon carcinoma xenograft in nude mice (n=5/group) at

doses of 50 mg/kg (Tween®-5109, previously shown as MTD) and 75 mg/kg (nab-5109) given q3d x 4, i.v. Control groups of PBS and human serum albumin (HSA) were also used.

[0223] Results: Nab-5109 yielded nanoparticles with mean size, Z_{Ave}=119 nm and Zeta potential = -32.7 mV. Nab-5109 was lyophilized to a dry powder that easily dispersed in saline. In vivo, there was significantly more weight loss (ANOVA, $p<0.001$) in the tumor bearing animals with Tween®-5109 (50mg/kg, 8.8% wt loss) than with nab-5109 (75mg/kg, 3.4% wt loss) indicating substantially lower toxicity of nab-5109 despite the 50% higher dose. There was significant tumor suppression by nab-5109 and Tween®-5109 (ANOVA, $p<0.0001$ vs. controls) with tumor growth delays of 36 and 28 days respectively for nab-5109 (75 mg/kg) and Tween®-5109 (50 mg/kg). Nab-5109 was more effective than Tween®-5109 (ANOVA, $p=0.0001$) in suppressing tumor growth. There were no differences between the PBS and HSA control group in term of toxicity and efficacy.

[0224] Conclusion: Nanoparticle albumin-bound, nab-5109 was successfully prepared and could be given at 50% higher dose than Tween®-5109 with lower toxicity despite higher dose. At this higher dose, 75 mg/kg (q3d x 4), nab-5109 showed significantly improved efficacy in the Pgp+ DLD-1 human colon xenograft compared with Tween®-5109.

Example 6. Nanoparticle Albumin Bound (*nab*) Dimeric Thiocolchicines nab-5404, nab-5800, and nab-5801: A Comparative Evaluation of Antitumor Activity vs Abraxane™ and Irinotecan

[0225] Methods: Nanoparticle colchicines were prepared using *nab* technology. Cytotoxicity was evaluated in vitro using human MX-1 breast carcinoma cultures. *In vivo* anti-tumor activity (human HT29 colon tumor xenograft) in nude mice was compared against Irinotecan and Abraxane™. Dose levels for the nab-colchicines and Irinotecan were 20 mg/kg, 30 mg/kg, and 40 mg/kg, given q3d x 4, i.v. Abraxane™ was dosed at its MTD, 30 mg/kg, given qd x 5.

[0226] Results: The hydrophobic thiocolchicine dimers yielded nanoparticles with average size Z_{Ave} (nm) of 119, 93, and 84 for nab-5404, nab-5800, and nab-5801, respectively. The nanoparticle suspensions were sterilized through 0.22 um filters and lyophilized. *In vitro*, nab-5404 was the most potent of the three analogs against MX-1 ($p \leq 0.0005$, ANOVA), (IC_{50} (ug/ml): 18, 36 and 77 for nab-5404, nab-5800 and nab-5801 respectively) as well as against the HT29 xenograft *in vivo* ($p \leq 0.0001$, ANOVA). Tumor

volume was suppressed by 93%, 79%, and 48% with nab-5404 at doses 40 mg/kg, 30 mg/kg, and 20 mg/kg, respectively. In contrast, tumor volume was only suppressed by 31%, 16%, and 21% with nab-5800; and 17%, 30%, and 23% with nab-5801 at 40 mg/kg, 30 mg/kg, and 20 mg/kg, respectively. Nab-5404 was more effective than Irinotecan at all dose levels ($p \leq 0.008$, ANOVA) with tumor volumes for Irinotecan suppressed by only 48%, 34%, and 29% at dose levels of 40 mg/kg, 30 mg/kg, and 20 mg/kg, respectively. In comparison to Abraxane™, nab-5404 was more active at equitoxic dose (ETD) based on equal weight loss ($p < 0.0001$, ANOVA). Tumor volume was suppressed 93% by nab-5404 (40 mg/kg, q4d x 3) and 80% by Abraxane™ (30 mg/kg, qd x 5) at their respective ETDs.

[0227] Conclusions: Nab technology was utilized to convert 3 hydrophobic dimeric thiocolchicines (IDN5404, IDN5800, IDN5801) to nanoparticles suitable for I.V. administration. Nab-5404 had superior antitumor activity in vitro and in vivo compared to nab-5800 and nab-5801. Nab-5404 was more potent than Irinotecan at equal dose. At equitoxic dose, defined by weight loss, nab-5404 was more potent than Abraxane™. These data warrant further investigation of nab-5404.

Example 7. Abraxane™ vs Taxotere®: A Preclinical Comparison of Toxicity and Efficacy

[0228] Methods: Toxicity of Abraxane™ and Taxotere® was compared in a dose-ranging study in nude mice given the drugs on a q4d x 3 schedule. The dose levels were Taxotere® 7, 15, 22, 33, and 50 mg/kg and ABX 15, 30, 60, 120, and 240 mg/kg. Antitumor activity of Abraxane™ and Taxotere® was compared in nude mice with human MX-1 mammary xenografts at a dose of 15 mg/kg weekly for 3 weeks.

[0229] Results: In the dose-escalation study in mice, the Taxotere® maximum tolerated dose (MTD) was 15 mg/kg and lethal dose (LD_{100}) was 50 mg/kg. In contrast, the Abraxane™ MTD was between 120 and 240 mg/kg and LD_{100} was 240 mg/kg. In the tumor study Abraxane™ was more effective than equal doses of Taxotere® in tumor growth inhibition (79.8% vs 29.1%, $p < 0.0001$, ANOVA).

[0230] Conclusion: Nanoparticle abumin-bound paclitaxel (Abraxane™) was superior to Taxotere® in the MX-1 tumor model when tested at equal doses. Furthermore, the toxicity of Abraxane™ was significantly lower than that of Taxotere®, which would allow dosing of Abraxane™ at substantially higher levels. These results are similar to the enhanced therapeutic index seen with Abraxane™ compared to Taxol® and suggest that the

presence of surfactants may impair the transport, antitumor activity and increase the toxicity of taxanes. Studies in additional tumor models comparing Abraxane™ and Taxotere® are ongoing.

Example 8. A Nanoparticle Albumin Bound Thiocolchicine dimer (nab-5404) with Dual Mechanisms of Action on Tubulin and Topoisomerase-1: Evaluation of *In vitro* and *In vivo* Activity

[0231] Methods: IDN5404 was tested for cytotoxic activity using the MCF7-S breast carcinoma and its multidrug resistant variant, MCF7-R (pgp+). Its cytotoxicity was also assessed against the NCI-60 human tumor cell line panel. The nanoparticle albumin bound nab-5404 was administered IV using various schedules, to SCID mice implanted s.c. with a human A121 ovarian tumor xenograft.

[0232] Results: Against MCF7 cell lines, the parent compound, colchicine, demonstrated tumor growth inhibition with the IC50 value (50% growth inhibitory concentration) for MCF7-S cells at 3.9 ± 0.2 nM. The resistant variant MCF7-R demonstrated an IC50 of 66 ± 8.6 nM, approximately a 17-fold increase due to drug resistance. IDN5404, demonstrated increased activity against both cell lines, displaying IC50 values of 1.7 ±0.1 and 40 ± 3.8 nM, respectively. These results were confirmed within the NCI 60 human tumor cell line panel with IDN5404 having a mean IC50 of $<10^{-8}$M and >10 fold resistance between the MCF7-S and the MCF7-R cell lines. The COMPARE algorithm identified IDN5404 as a tubulin binder similar to vinca alkaloids, confirming the previous results. *In vivo* against the A121 ovarian tumor xenograft, efficacy and toxicity of nab-5404 was dose and schedule dependent. Nanoparticle nab-5404 was well tolerated and capable of inducing complete regressions and cures: at 24 mg/kg administered IV qd x 5, 5 of 5 mice were long-term survivors (LTS) with no evidence of tumor. However, increasing the dosage to 30 mg/kg resulted in 5 of 5 toxic deaths. On a q3d x 4 schedule, 30 mg/kg resulted in 4 of 5 mice LTS and at 50 mg/kg, 5 of 5 toxic deaths. Using a q7d x 3 schedule, 40 mg/kg resulted in 3 of 5 mice LTS and at 50 mg/kg, 4 of 4 LTS were noted.

[0233] Conclusions: IDN5404, a new thiocolchicine dimer with dual mechanism of action showed activity in pgp-expressing, cisplatin and topotecan resistant cell lines. *In vivo*, nanoparticle albumin bound nab-5404 was active against A121 ovarian xenografts.

Example 9. Combination Studies of Abraxane™ and Other Agents

[0234] Due to the advantageous properties of Abraxane™ (ABX, the nanoparticle albumin-bound paclitaxel) noted above, it was used and being used in a number of studies with different modes of administration and schedules and in combination with other oncology drugs as well as radiation treatment. These are listed below:

[0235] In metastatic breast cancer, these studies include:

Randomized Phase II Trial of Weekly Abraxane™ in Combination with Gemcitabine in Individuals with HER2 Negative Metastatic Breast Cancer	ABX 125, Gem 1000 mg/m^2, D1,8; q 3wk	To evaluate the combination of ABX and Gemcitabine in 1st-line MBC.
A phase II study of weekly dose-dense nanoparticle paclitaxel (ABI-007) carboplatin, with Herceptin® as first or second-line therapy of advanced HER2 positive breast cancer	ABX 100 mg/m^2, Carbo AUC 2, both D1,8,15; Her 2 mg/kg (4 mg/kg on wk a) q4wk x 6	Data will be important for using ABX in combination with carbo and/or Herceptin®. Also helpful for other combinations.
Weekly Vinorelbine and Abraxane™, with or without G-CSF, in stage IV breast cancer: a phase I-II study	L1: ABX 80, Nav 15; L2: ABX 90, Nav 20; L3: ABX 100, Nav 22.5; L4: ABX 110, Nav 25; L5: ABX 125, Nav 25 qwk	Multi-center study of ABX in combination with Navelbine® in 1st-line MBC.
Phase II trial of weekly Abraxane™ monotherapy for 1st-line MBC (plus Herceptin® in Her2+ pts)	ABX 125 mg/m^2 Q3/4wk	A relatively large phase II of weekly ABX monotherapy at 125 mg/m^2 in 1st-line MBC.
Phase I/II trial Abraxane™ plus Doxil® for MBC plus limited PK	ABX + Anthracycline	
3-arm phase II trial in 1st-line MBC	ABX weekly (130 mg/m^2) vs. q2wk (260 mg/m^2) vs. q3wk (260 mg/m^2)	To optimize ABX monotherapy regime for MBC
3-arm phase II trial in 1st-line and 2nd-line MBC, with biological correlates analyses	ABX weekly vs. ABX q3wk vs. Taxol® weekly	randomized ABX MBC trial to obtain important data: weekly ABX vs. weekly Taxol®; weekly ABX vs. 3-weekly ABX; plus biomarker study (caveolin-1 and SPARC).
Phase I/II Abraxane™ + GW572016	TBD	combination of ABX and GW572016 (a dual EGFR inhibitor and one of the most promising new biological agents for BC).
A phase I dose escalation study of a 2 day oral gefitinib chemosensitization pulse given prior to weekly Abraxane™ in individuals with advanced solid tumors	Abraxane™ 100 mg/m^2 weekly, 3 out of 4 weeks; Gefitinib starting at 1000 mg/d x 2 days	This phase I trial is to determine the safety and tolerability of a 2 day gefitinib pulse given prior to Abraxane™ administration.

Phase II 1st line MBC trial	weekly ABX (125 mg/m^2, 2 wk on and 1 wk off) + Xeloda® 825 mg/m^2 d 1-14 q3wk	To evaluate the combination of ABX and Xeloda® in 1st-line MBC, using 2 weekly on and 1 weekly off ABX regime.
Phase II pilot adjuvant trial of Abraxane™ in breast cancer	Dose dense AC + G CSF --> weekly ABX --> Avastin®	A pilot adjuvant study of a "super dose dense"
Abraxane™ in dose-dense adjuvant chemotherapy for early stage breast cancer	AC q2w x 4 + G CSF --> ABX q2wk x 4	A pilot adjuvant study of dose dense ABX regime -- an alternate of a standard adjuvant regime
Phase II pilot adjuvant trial of Abraxane™ in breast cancer	AC Q2wk --> ABX q2wk + G-CSF	A pilot adjuvant study in preparation for phase III adjuvant trial

[0236] In Breast cancer neoadjuvant setting studies include:

Phase II Trial of Dose Dense Neoadjuvant Gemcitabine, Epirubicin, ABI-007 (GEA) in Locally Advanced or Inflammatory Breast Cancer	Neoadjuvant: Gem 2000, Epi 60, ABX 175 mg/m^2, Neul 6 mg SC, all D1 q2 wk x 6 Adjuvant: Gem 2000, ABX 220, Neul 6 mg D1 q2wk x 4	This neoadjuvant study is based on the GET data from Europe which showed high activity. In the current regime, ABX will replace T, or Taxol®.
Phase II preoperative trial of Abraxane™ followed by FEC (+ Herceptin® as appropriate) in breast cancer	ABX 220 mg/m^2 q2wk x 6 followed by FEC x 4 (+Herceptin® for Her2+ pts)	
Pre-clinical study of drug-drug interaction	ABX + other agents	
Phase II neoadjuvant	(ABX + Herceptin®) followed by (Navelbine® + Herceptin®)	
Randomized phase II trial of neoadjuvant chemotherapy in individuals with breast cancer	TAC vs. AC followed ABX+carbo vs. AC followed ABX+carbo+Herceptin®	To evaluate AC followed by ABX/carbo or ABX/carbo/Herceptin® combinations vs TAC (a FDA approved adjuvant BC regime) in neoadjuvant setting.
Phase II neoadjuvant trial of Abraxane™ and capecitabine in locally advanced breast cancer	ABX: 200 mg/m^2 D1; Xel: 1000 mg/m^2 D1-14; q3wk x 4	
Phase II trial of neoadjuvant chemotherapy (NCT) with nanoparticle paclitaxel (ABI-007, Abraxane™) in women with clinical stage IIA, IIB, IIIA, IIIB, and IV (with intact primary) breast cancers	ABX: 300 mg/m^2 q3wk	

[0237] In lung cancer the studies include:

Phase I/II study of Abraxane™ monotherapy in 1st-line advanced NSCLC	ABX weekly	The first phase II trial of ABX combo with carbo in NSCLC.
Phase II Trial of weekly Abraxane™ plus carboplatin in 1st-line NSCLC	ABX: 125mg/m^2 D1,8,15; Carbo: AUC 6 D1; q4 wk	
A Phase I Trial of Carboplatin and Abraxane™ on a weekly and every three week schedule in individuals with Advanced Solid Tumor Malignancies	Arm 1: ABX 100, 125, 150 mg/m^2 D1,8,15 q4wk; Arm 2: ABX 220, 260, 300 mg/m^2 D1 q3wk. Carbo AUC6 in both arms	This 2-arm phase I study will generate important data on ABX/carbo combination for further studies of this combo in multiple diseases.
Phase II study of ABI 007 (Abraxane™) and carboplatin in advanced non-small cell lung cancer.	ABX Level(a): 225 mg/m2; Level(b): 260 mg/m2; Level(3):300 mg/m2;q3wk Carbo fixed at AUC6 q3wk	This phase II NSCLC study will generate data for a future phase III registration trial in lung cancer
Phase I study of ABI 007 (Abraxane™) and carboplatin	ABX q3wk	
Phase I/II study of Abraxane™ + Alimta® for 2nd-line NSCLC	TBD	ABX and Alimta® can be a promising combination due to the non-overlapping toxicity profiles.
Phase I/II trial of Abraxane™ plus cisplatin in advanced NSCLC		
Phase I/II study of Abraxane™, Navelbine®, and Cisplatin for treatment of advanced NSCLC		
Phase II ABX mono in 1st-line NSCLC	ABX 260 mg/m^2 q3wk	The 1st ABX trial in NSCLC.

Phase II study of Abraxane™ monotherapy in 2nd-line NSCLC	Cohort 1: ABX q3wk; Cohort 2: ABX weekly. Doses TBD	
Phase I/II trial of weekly Abraxane™ and carboplatin in advanced NSCLC	1st line	

[0238] Studies in Prostate include:

Randomized phase II ABX weekly vs Q3W in front line HRP	100 mg/m^2 weekly vs 260 mg/m^2 q3wk	
Phase II ABX in 1st-line prostate cancer	weekly ABX	Phase II study of weekly ABX in 1st-line HRPC
Phase II neoadjuvant study	TBD	A multi-center neoadjuvant trial of ABX in prostate cancer plus biomarker study.
Phase II ABX 100 mg weekly no break		

[0239] Studies in ovarian cancer include:

Phase II study of Abraxane™ for treatment of advanced ovarian cancer (3rd-line)	TBD	
Phase I study of Abraxane™ plus carbo for treatment of advanced ovarian cancer	ABX weekly + Carbo AUC 6	
A phase II trial of Abraxane™/Carboplatin in recurrent ovarian cancer		

[0240] Studies in Chemoradiation include:

Phase I/II trial of Abraxane™ combined with radiation in NSCLC		
Abraxane™ Combined With Radiation	animal model	
H&N (Head and Neck Cancer)	TBD	

[0241] Other studies include:

Phase II study of ABX in treatment of persistent or recurrent carcinoma of the cervix	125 mg/m^2 d1,8,15 q28 days	
PhII in preciously treated (100 ABX) and untreated (150 ABX) metastatic melanoma	26-->70	
Ph II single treatment use of ABI-007 for the treatment of non-hematologic malignancies		
Abraxane™ Combined With antiangiogenic agents, e.g., Avastin®.		
Abraxane™ Combined With proteasome inhibitors e.g., Velcade®.		
Abraxane™ Combined With EGFR inhibitors e.g., Tarceva®.		
A randomized phase II trial of weekly gemcitabine, Abraxane™, and external irradiation for locally advanced pancreatic cancer		

Example 10. Combination of nanoparticle invention drugs with other agents and modes of therapy.

[0242] Lower toxicity of nanoparticle invention drugs described herein allow combination with other oncology drugs and other modes of treatment with more advantageous outcome. These include nanoparticle forms of paclitaxel, docetaxel, other taxanes and analogs, geldanamycins, colchicines and analogs, combretastatins and analogs, hydrophobic pyrimidine compounds, lomaiviticins and analogs including compounds with the lomaiviticin core structures, epothilones and analogs, discodermolide and analogs and the like. The invention drugs may be combined with paclitaxel, docetaxel, carboplatin, cisplatin, other platins, doxorubicin, epirubicin, cyclophosphamide, iphosphamide, gemcitabine, capecitabine, vinorelbine, topotecan, irinotecan, tamoxifen, camptothecins, 5-FU, EMP, etoposide, methotraxate and the like.

Example 11. Combination of Abraxane™ with Carboplatin and Herceptin®

[0243] The combination of Taxol® and carboplatin has shown significant efficacy against metastatic breast cancer. On a weekly schedule, in this combination, Taxol® can only be dosed at up to 80mg/m^2. Higher doses cannot be tolerated due to toxicity. In addition, HER-2-positive individuals derive greater benefit when Herceptin® is included in their therapeutic regime. This open-label Phase II study was conducted to determine the synergistic therapeutic effect of ABI-007 (Abraxane™) with these agents. The current study was initiated to evaluate the safety and antitumor activity of ABI-007/carboplatin with Herceptin® for individuals with HER-2 positive disease. ABI-007 was given in combination with carboplatin and Herceptin® administered intravenously weekly to individuals with HER-2 positive advanced breast cancer. A cohort of 3 individuals received ABI-007 at a dose of 75 mg/m^2 IV followed by carboplatin at target AUC = 2 weekly and Herceptin® infusion (4 mg/kg at week 1, and 2 mg/kg on all subsequent weeks) for 1 cycle. These individuals tolerated the drug very well so for all subsequent cycles and individuals the dose of ABI-007 was escalated to 100 mg/m^2. Six individuals were treated to date. Of the 4 individuals that were evaluated for response, all 4 (100%) showed a response to the therapy. It should be noted that due to lower toxicity of Abraxane™, a higher total paclitaxel dose could be given compared to Taxol® with resulting benefits to the individuals.

Example 12. Combination of Abraxane™ with Carboplatin

[0244] The combination of Taxol® and carboplatin has shown significant efficacy in lung cancer. Another study with Abraxane™ in combination with carboplatin on a 3 weekly schedule in individuals with lung cancer is ongoing.

Example 13. Use of Abraxane™ in Combination With Radiation

Example 13a

[0245] Abraxane™, combined with clinical radiotherapy, enhances therapeutic efficacy and reduces normal tissue toxicity. Abraxane™ is used to increase the therapeutic gain of radiotherapy for tumors; to enhance tumor response to single and fractionated irradiation; to enhance normal tissue response to radiation and to increase therapeutic ratio of radiotherapy.

[0246] A murine ovarian carcinoma, designated OCa-I, which has been investigated extensively is used. First, optimal timing of Abraxane™ administration relative to local tumor radiation is timed to produce maximum antitumor efficacy. Tumors are generated in the right hind leg of mice by i.m. injection of tumor cells and treatment is initiated when the tumors reach 8mm in size. Mice are treated with 10 Gy single dose irradiation, a single dose of Abraxane™ or with combination therapy of Abraxane™ given at different times 5 days before to 1 day after irradiation. A dose of Abraxane™ equal to about 1½ times more than the maximum tolerated dose of paclitaxel is used, a dose of 90 mg/kg. The endpoint of efficacy is tumor growth delay. The groups consist of 8 mice each. Tumors are generated and treated as described in Aim 1. The endpoint of efficacy is tumor growth delay. Tumors are irradiated with 5, 7.5 or 10 Gy delivered either in a single dose or in fractionated doses of 1, 1.5 or 2 Gy radiation daily for five consecutive days. Since Abraxane™ is retained in the tumor for several days and exerts its enhancing effect on each of the five daily fractions, Abraxane™ is given once at the beginning of the radiation regime. Since the ultimate goal in clinical radiotherapy is to achieve tumor cure, the potential for Abraxane™ to enhance tumor radiocurability is determined. The same scheme as described for the fractionated tumor growth delay study is used, except that a range of doses from 2 to 16 Gy is given daily for five consecutive days (total radiation dose 10 to 80 Gy). Tumors are followed for regression and regrowth for up to 120 days after irradiation, when TCD50 (the dose of radiation needed to yield local tumor cure in 50 percent of animals) is determined. There are two TCD50 assays: radiation only and Abraxane™ plus radiation, and each assay consists of 10 radiation dose groups containing 15 mice each. To provide therapeutic gain, any radioenhancing agent, including Abraxane™, must increase tumor radioresponse more than increase normal tissue damage by radiation. Damage to jejunal mucosa, a highly proliferative tissue affected by taxanes is assessed. The jejunal microcolony assay is used to determine the survival of crypt epithelial cells in the jejunum of mice exposed to radiation. Mice are exposed to whole body irradiation (WBI) with daily doses of X-rays ranging from 3 to 7 Gy for five consecutive days. The mice are treated with Abraxane™, at an equivalent paclitaxel dose of 80 mg/kg, administered i.v. 24 h before the first dose of WBI and killed 3.5 days after the last dose of WBI. The jejunum is prepared for histological examination, and the number of regenerating crypts in the jejunal cross-section is counted. To construct radiation survival curves, the number of regenerating crypts is converted to the number of surviving cells.

Example 13b

[0247] The objective of this study was to assess whether ABI-007 (a) as a single agent has antitumor activity against the syngeneic murine ovarian carcinoma OCa-1 and (b) enhances the radiation response of OCa-1 tumors in a combined treatment regime as described in the previous example with the following modifications.

[0248] OCa-1 tumor cells were injected i.m. into the hind leg of C3H mice. When tumors grew to a mean diameter of 7 mm, single treatment with local radiation (10 Gy) to the tumor-bearing leg, ABI-007 90 mg/kg i.v., or both, was initiated. To determine the optimal treatment scheduling, ABI-007 was given from 5 days to 9 hours before radiation as well as 24 hours after radiation. Treatment endpoint was absolute tumor growth delay (AGD), defined as the difference in days to grow from 7-12 mm in diameter between treated and untreated tumors. For groups treated with the combination of ABI-007 and radiation, an enhancement factor (EF) was calculated as the ratio of the difference in days to grow from 7 to 12 mm between the tumors treated with the combination and those treated with ABI-007 alone to the AGD of tumors treated with radiation only. To assess the radiation-enhancing effect of ABI-007 for a fractionated radiation regime on the endpoint tumor cure, a TCD50 assay was performed and analyzed 140 days post treatment. Total doses of 5 to 80 Gy in 5 daily fractions were administered either alone or combined with ABI-007 24 hours before the first radiation dose.

[0249] As a single agent, ABI-007 significantly prolonged the growth delay of the OCa-1 tumor (37 days) compared to 16 days for untreated tumors. ABI-007 as a single agent was more effective than a single dose of 10 Gy, which resulted in a delay of 29 days. For combined treatment regimes, ABI-007 given at any time up to 5 days before radiation, produced a supra-additive antitumor effect. EF was 1.3, 1.4, 2.4, 2.3, 1.9, and 1.6 at intertreatment intervals of 9h, 24 h and 2, 3, 4, and 5 days, respectively. When ABI-007 was given after radiation, the combined antitumor treatment effect was less than additive. Combined treatment with ABI-007 and radiation also had a significant effect on tumor cure by shifting the TCD50 of 55.3 Gy for tumors treated with radiation only to 43.9 Gy for those treated with the combination (EF 1.3).

[0250] This experiment demonstrated that ABI-007 possesses single-agent antitumor activity against OCa-1 and enhances the effect of radiotherapy when given several days prior. As previously demonstrated for paclitaxel and docetaxel, the radiation

enhancement is likely a result of multiple mechanisms, with a cell cycle arrest in G2/M being dominant at short treatment intervals and tumor reoxygenation at longer intervals.

Example 14. Combination of Abraxane™ and Tyrosine Kinase Inhibitors

[0251] Pulse-dosing of gefitinib in combination with the use of Abraxane™ is useful to inhibit the proliferation of EGFr expressing tumors. 120 nude mice are inoculated with BT474 tumor cells to obtain at least 90 mice bearing BT474 xenograft tumors and split into 18 experimental arms (5 mice each). Arm 1 mice receive control i.v. injections. All other mice receive weekly i.v. injections of Abraxane™ at 50 mg/kg for 3 weeks. Arm 2 receive Abraxane™ alone. Arms 3, 4, 5, 6, 7, 8 receive weekly Abraxane™ preceded by 2 days of a gefitinib pulse at increasing doses. Arms 9, 10, 11, 12, 13 receive weekly Abraxane™ preceded by one day of a gefitinib pulse at increasing doses. Arms 14, 15, 16, 17, 18 receive weekly Abraxane™ along with everyday administration of gefitinib at increasing doses. The maximum tolerated dose of gefitinib that can be given in a 1 or 2 day pulse preceding weekly Abraxane™ or in continuous administration with Abraxane™ is established. In addition, measurement of anti-tumor responses will determine whether a dose-response relationship exists and whether 2 day pulsing or 1 day pulsing is superior. These data are used to select the optimal dose of pulse gefitinib and that of continuous daily gefitinib given with Abraxane™.

[0252] 120 nude mice are inoculated with BT474 tumor cells to obtain 90 mice bearing tumors. These mice are split into 6 groups (15 each). Arm 1 receive control i.v. injections. Arm 2 receive Abraxane™ 50 mg/kg i.v. weekly for 3 weeks. Arm 3 receive oral gefitinib at 150 mg/kg/day. Arm 4 receive Abraxane™ 50 mg/kg along with daily gefitinib at the previously established dose. Arm 5 receive Abraxane™ 50 mg/kg preceded by a gefitinib pulse at the previously established dose and duration. Arm 6 receive only a weekly gefitinib pulse at the previously established dose. After three weeks of therapy, mice are followed until controls reach maximum allowed tumor sizes.

Example 15. Phase II Study of Weekly, Dose-dense *nab*™-Paclitaxel (Abraxane™), Carboplatin With Trastuzumab® As First-line Therapy Of Advanced HER-2 Positive Breast Cancer

[0253] This study aimed to evaluate (1) the safety and tolerability and (2) the objective response rate of weekly dose-dense trastuzumab/Abraxane™/carboplatin as first-line cytotoxic therapy for patients with advanced/metastatic (Stage IV adenocarcinoma)

HER-2-overexpressing breast cancer. Trastuzumab is a monoclonal antibody, also known as Herceptin®, which binds to the extracellular segment of the erbB2 receptor.

[0254] Briefly, patients without recent cytotoxic or radiotherapy were included. Doses of Abraxane™ were escalated from 75 mg/m^2 as 30-min i.v. infusions on days 1, 8, 15 up to 100 mg/m^2 for subsequent cycles according to the standard 3 + 3 rule. Carboplatin AUC = 2 was given as 30-60 min i.v. infusions on days 1, 8, 15 and for an initial 29 day cycle. Trastuzumab was given as i.v. 30-90 min infusion on days 1, 8, 15, 22 at a dose of 4 mg/kg at week 1 and 2 mg/kg on all subsequent weeks.

[0255] Of 8 out of 9 patients evaluable for response the response rate (confirmed plus unconfirmed) was 63% with 38% stable disease. The most common toxicities were neutropenia (grade 3: 44%; grade 4: 11%) and leukocytopenia (33%).

[0256] These results suggest that trastuzumab plus Abraxane™ plus carboplatin demonstrated a high degree of antitumor activity with acceptable tolerability as a first-line therapy for MBC.

Example 16. Phase II Trial of Capecitabine Plus *nab*™-Paclitaxel (Abraxane™) in the First Line Treatment of Metastatic Breast Cancer

[0257] The purpose of this phase II study was to evaluate the safety, efficacy (time to progression and overall survival), and quality of life of patients with MBC who received capecitabine in combination with Abraxane™. Capecitabine is a fluoropyrimidine carbamate also known as Xeloda® which has been shown to have substantial efficacy alone and in combination with taxanes in the treatment of MBC.

[0258] In this open-label, single-arm study, Abraxane™ 125 mg/m^2 was given by i.v. infusion on day 1 and day 8 every 3 weeks plus capecitabine 825 mg/m^2 given orally twice daily on days 1 to 14 every 3 weeks. Patients were HER-2/neu negative with a life expectancy of greater than 3 months. Patients had no prior chemotherapy for metastatic disease, no prior capecitabine therapy, and no prior fluoropyrimidine therapy and paclitaxel chemotherapy given in an adjuvant setting.

[0259] 12 patients have been enrolled with safety analysis completed on the first 6 patients and the response rate evaluable after 2 cycles in the first 8 patients. There were no unique or unexpected toxicities with no grade 4 toxicities or neuropathy greater than grade 1. Response data were confirmed on only the first 2 cycles of therapy (first evaluation point) in 6 patients. Two patients have completed 6 cycles with 1 partial response and 1

stable disease. Of the first 8 patients after 2 cycles, there were 2 partial responses and 4 with stable disease.

[0260] These results show that combination of capecitabine and weekly Abraxane™ at effective doses is feasible with no novel toxicities to date. Abraxane™ related toxicity was mainly neutropenia without clinical consequences, and hand foot syndrome was the major toxicity of capecitabine.

Example 17. Pilot Study of Dose-Dense Doxorubicin Plus Cyclophosphamide Followed by *nab*-paclitaxel (Abraxane™) in Patients with Early-Stage Breast Cancer

[0261] The objective of this study was to evaluate the toxicity of doxorubicin (adriamycin) plus cyclophosphamide followed by Abraxane™ in early stage breast cancer.

[0262] Patients had operable, histologically confirmed breast adenocarcinoma of an early stage. The patients received doxorubicin (adriamycin) 60 mg/m^2 plus cyclophosphamide 600 mg/m^2 (AC) every 2 weeks for 4 cycles followed by Abraxane™ 260 mg/m^2 every two weeks for 4 cycles.

[0263] 30 patients received 4 cycles of AC, and 27 of 29 patients received 4 cycles of Abraxane™; 33% of patients received pegfilgrastim (Neulasta®) for lack of recovery of ANC (absolute neutrophil count) during Abraxane™. Nine patients (31%) had Abraxane™ dose reductions due to non-hematologic toxicity. A total of 9 patients had grade 2 and 4 patients had grade 3 peripheral neuropathy (PN); PN improved by ≥1 grade within a median of 28 days.

[0264] These results indicate that dose-dense therapy with doxorubicin (60 mg/m^2) plus cyclophosphamide (600 mg/m^2) every 2 weeks for 4 cycles followed by dose-dense Abraxane™ (260 mg/m^2) every 2 weeks for 4 cycles was well tolerated in patients with early-stage breast cancer.

Example 18. Weekly *nab*-Paclitaxel (Abraxane™) as First Line Treatment of Metastatic Breast Cancer with Trastuzumab Add On for HER-2/*neu*-Positive Patients

[0265] The purpose of the current study was to move weekly Abraxane to a front-line setting and add trastuzumab for HER2/*neu*-positive patients.

[0266] This phase II, open-label study included 20 HER2-postivive and 50 HER2-negative patients with locally advanced or metastatic breast cancer. Abraxane™ was given at 125 mg/m^2 by 30 minute i.v. infusion on days 1, 8, and 15 followed by a week of rest. Trastuzumab was given concurrently with study treatment for patients who were HER2-

positive. The primary endpoint was response rate and the secondary endpoints were time to progression (TTP), overall survival (OS), and toxicity.

[0267] In the safety population, 23 patients received a median of 3 cycles of Abraxane™ to date. The most common treatment-related adverse event was grade 3 neutropenia (8.7%) with no grade 4 adverse events. One out of 4 evaluable patients responded to therapy.

Example 19. Phase I Trial of *nab*-Paclitaxel (Abraxane™) and Carboplatin

[0268] The aim of the current study was to determine the maximum tolerated dose of Abraxane™ (both weekly and every 3 weeks) with carboplatin AUC = 6 and to compare the effects of sequence of administration on pharmacokinetics (PK).

[0269] Patients with histologically or cytologically documented malignancy that progressed after "standard therapy" were included. Arm 1 received Abraxane™ every 3 weeks in a dose escalation format based on cycle 1 toxicities (220, 260, 300, 340 mg/m^2) every 3 weeks followed by carboplatin AUC = 6. Arm 2 received weekly (days 1, 8, 15 followed by 1 week off) Abraxane™ (100, 125, 150 mg/m^2) followed by carboplatin AUC = 6. For the PK portion of the study, Abraxane™ was followed by carboplatin in cycle 1 and the order of administration reversed in cycle 2 with PK levels determined at initial 6, 24, 48 and 72 hours.

[0270] On the every 3 weeks schedule, neutropenia, thrombocytopenia and neuropathy were the most common grade 3/4 toxicities (3/17 each). On the weekly schedule, neutropenia 5/13 was the most common grade 3/4 toxicity. The best responses to weekly administration at the highest dose of 125 mg/m^2 (n = 6) were 2 partial responses (pancreatic cancer, melanoma) and 2 stable disease (NSCLC). The best responses to the every three week administration at the highest dose of 340 mg/m^2 (n = 5) were 1 stable disease (NSCLC) and 2 partial responses (SCLC, esophageal).

[0271] These data indicate activity of combination of Abraxane™ and carboplatin. The MTD for the weekly administration was 300 mg/m^2, and for the once every 3 week administration was 100 mg/m^2.

Example 20. Phase II Trial of Dose-Dense Gemcitabine, Epirubicin, and *nab*-Paclitaxel (Abraxane™) (GEA) in Locally Advanced/Inflammatory Breast Cancer

[0272] In an open-label, phase II study an induction/neoadjuvant therapy regime was instituted prior to local intervention. The therapy regime was gemcitabine 2000 mg/m^2

i.v. every 2 weeks for 6 cycles, epirubicin 50 mg/m^2 every 2 weeks for 6 cycles, Abraxane™ 175 mg/m^2 every 2 weeks for 6 cycles, with pegfilgrastim 6 mg s.c. on day 2 every 2 weeks. The postoperative/adjuvant therapy regime after local intervention was gemcitabine 2000 mg/m^2 every 2 weeks for 4 cycles, Abraxane™ 220 mg/m^2 every 2 weeks for 4 cycles and pegfilgrastim 6 mg s.c. day every 2 weeks. Patients included females with histologically confirmed locally advanced/inflammatory adenocarcinoma of the breast.

Example 21. Cytotoxic activity of *nab*-rapamycin in combination with Abraxane™ on vascular smooth muscle cells

[0273] Vascular smooth muscle cells (VSMC) were seeded onto 96 wells plates in the presence of increasing concentrations of *nab*-rapamycin and 0 μM, 1 μM, 10 μM, or 100 μM of Abraxane™ (ABI-007). To evaluate the cytotoxic effect of nab-rapamycin and Abraxane™, treated VSMCs were stained with ethidium homodimer-1 (Invitrogen, Carlsbad CA) and analyzed for red fluorescence. Ethidium homodimer-1 is a high-affinity, fluorescent nucleic acid stain that is only able to pass through compromised membranes of dead cells to stain nucleic acids. As shown in Fig. 7A, *nab*-rapamycin, by itself, exhibited dose-dependent cell killing as demonstrated by increasing fluorescence. Cell killing by nab-rapamycin was not enhanced by Abraxane™ at 1 μM or 10 μM; however, it was greatly enhanced by Abraxane™ at 100 μM (ANOVA, $p < 0.0001$). Cells stained with ethidium homodimer-1 as shown in Fig. 7A were also exposed to calcein. Calcein AM (Invitrogen) is a non-fluorescent molecule that is hydrolyzed into fluorescent calcein by nonspecific cytosolic esterases. Live cells exposed to calcein AM exhibit bright green fluorescence as they are able to generate the fluorescent product and retain it. As shown in Fig. 7B, nab-rapamycin exhibited dose dependent cytotoxic activity as shown by a reduced amount of fluorescent staining by calcein. This reduction in fluorescence was enhanced by coincubation with Abraxane™ in a dose dependent manner. ANOVA statistic gave $p < 0.0001$ at all drug concentrations of Abraxane™.

Example 22. Cytotoxic activity of nab-rapamycin in combination with Abraxane™ against HT29 (human colon carcinoma) tumor xenograft.

[0274] Nude mice were implanted with 10^6 HT29 cells on their right flanks. Treatment was initiated once the tumor were palpable and were greater than 100-200 mm^3. The mice were randomly sorted into 4 groups (n= 8 per group). Group 1 received saline 3 times weekly for 4 weeks, i.v.; Group 2 received Abraxane™ at 10 mg/kg, daily for 5 days, i.p.; Group 3 received nab-rapamycin at 40 mg/kg, 3 times weekly for 4 weeks, i.v.; and Group 4 received both nab-rapamycin (40 mg/kg, 3 times weekly for 4 weeks, i.v.) and Abraxane™ (10 mg/kg, daily for 5 days, i.p.). As shown in Fig. 8, the tumor suppression was greater for the Abraxane™ plus nab-rapamycin combination therapy than for either single therapy group.

Example 23. Cytotoxic activity of nab-17-AAG in combination with Abraxane™ against H358 (human lung carcinoma) tumor xenograft.

[0275] Nude mice were implanted with 10^7 H358 cells on their right flanks. Treatment was initiated once the tumors were palpable and were greater than 100-200 mm^3. The mice were randomly sorted into 4 groups (n= 8 per group). Group 1 received saline 3 times weekly for 4 weeks, i.v.; Group 2 received Abraxane™ at 10 mg/kg, daily for 5 days, i.p.; Group 3 received nab-17-AAG at 80 mg/kg, 3 times weekly for 4 weeks, i.v.; and Group 4 received both nab-17-AAG (80 mg/kg, 3 times weekly for 4 weeks, i.v.) and Abraxane™ (10 mg/kg, daily for 5 days, i.p.). As shown in Fig. 9, the tumor suppression was greater for the nab-17-AAG plus Abraxane™ combination therapy than for either single therapy group.

[0276] Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it is apparent to those skilled in the art that certain minor changes and modifications will be practiced. Therefore, the description and examples should not be construed as limiting the scope of the invention.

[0277] All references, including publications, patent applications, and patents, cited herein are hereby incorporated by reference to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein.

[0278] Preferred embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those preferred embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such

variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

CLAIMS

What is claimed is:

1. A method of treating a proliferative disease in an individual comprising administering to the individual:
a) an effective amount of a composition comprising nanoparticles comprising a taxane and an albumin, and
b) an effective amount of at least one other chemotherapeutic agent, wherein said chemotherapeutic agent is selected from the group consisting of antimetabolites, platinum-based agents, alkylating agents, tyrosine kinase inhibitors, anthracycline antibiotics, vinca alkloids, proteasome inhibitors, macrolides, and topoisomerase inhibitors.

2. The method according to claim 1, wherein said chemotherapeutic agent is an antimetabolite agent.

3. The method according to claim 2, wherein said antimetabolite agent is gemcitabine, capecitabine, or fluorouracil.

4. The method according to claim 1, wherein said chemotherapeutic agent is a platinum-based agent.

5. The method according to claim 4, wherein said platinum-based agent is cisplatin or carboplatin.

6. The method according to claim 1, wherein said chemotherapeutic agent is a tyrosine kinase inhibitor.

7. The method according to claim 6, wherein said tyrosine kinase inhibitor is lapatinib.

8. The method according to claim 1, wherein the proliferative disease is cancer.

9. The method according to claim 8, wherein the cancer is breast cancer.

10. The method according to claim 8, wherein the cancer is lung cancer.

11. The method according to claim 1, wherein the composition comprising nanoparticles comprising taxane and albumin and the chemotherapeutic agent are administered simultaneously.

12. The method according to claim 1, wherein the composition comprising nanoparticles of taxane comprising albumin and the chemotherapeutic agent are administered sequentially.

13. The method according to claim 1, wherein the taxane is paclitaxel.

14. The method according to claim 13, wherein the average diameter of the nanoparticles in the composition is no greater than about 200 nm.

15. The method according to claim 14, wherein the weight ratio of the albumin and the paclitaxel in the nanoparticle composition is less than about 9:1.

16. The method according to claim 14, wherein the nanoparticle composition is substantially free of Cremophor.

17. The method according to claim 1, wherein the average diameter of the nanoparticles in the composition is no greater than about 200 nm.

18. The method according to claim 17, wherein the weight ratio of the albumin and the taxane in the nanoparticle composition is less than about 9:1.

19. The method according to claim 17, wherein the nanoparticle composition is substantially free of Cremophor.

20. The method according to claim 1, wherein the individual is a human.

21. A method of treating a tumor in an individual comprising:
a) a first therapy comprising administering to the individual an effective amount of a composition comprising nanoparticles comprising a taxane and an albumin, and

b) a second therapy comprising radiation therapy, surgery, or combinations thereof.

22. The method of claim 21, wherein the second therapy is radiation therapy.

23. The method of claim 22, wherein the radiation therapy occurs before the first therapy.

24. The method of claim 21, wherein the second therapy is surgery.

25. The method according to claim 21, wherein the taxane is paclitaxel.

26. The method according to claim 25, wherein the average diameter of the nanoparticles in the composition is no greater than about 200 nm.

27. The method according to claim 26, wherein the weight ratio of the albumin and the paclitaxel in the nanoparticle composition is less than about 9:1.

28. The method according to claim 26, wherein the nanoparticle composition is substantially free of Cremophor.

29. The method according to claim 21, wherein the average diameter of the nanoparticles in the composition is no greater than about 200 nm.

30. The method according to claim 29, wherein the weight ratio of the albumin and the taxane in the nanoparticle composition is less than about 9:1.

31. The method according to claim 29, wherein the nanoparticle composition is substantially free of Cremophor.

32. The method according to claim 21, wherein the individual is a human.

33. A method of administering a composition comprising nanoparticles comprising a taxane and an albumin to an individual,
wherein the nanoparticle composition is administered over a period of at least one month,
wherein the interval between each administration is no more than about a week, and

wherein the dose of taxane in the composition at each administration is about 0.25% to about 25% of its maximum tolerated dose following a traditional dosing regime.

34. The method according to claim 33, wherein the dose of taxane is about 0.25% to about 8% of its maximum tolerated dose following a traditional dosing regime.

35. The method according to claim 33, wherein the composition is administered at least three times a week.

36. The method according to claim 33, wherein said taxane is paclitaxel.

37. The method according to claim 36, wherein the average diameter of the nanoparticles in the composition is no greater than about 200 nm.

38. The method according to claim 37, wherein the weight ratio of the albumin and the paclitaxel in the nanoparticle composition is less than about 9:1.

39. The method according to claim 37, wherein the nanoparticle composition is substantially free of Cremophor.

40. The method according to claim 33, wherein the average diameter of the nanoparticles in the composition is no greater than about 200 nm.

41. The method according to claim 40, wherein the weight ratio of the albumin and the taxane in the nanoparticle composition is less than about 9:1.

42. The method according to claim 40, wherein the nanoparticle composition is substantially free of Cremophor.

43. The method according to claim 33, wherein the individual is a human.

44. A method of treating a proliferative disease in an individual, comprising administering to the individual: a) an effective amount of a composition comprising nanoparticles comprising a taxane and an albumin; and b) an effective amount of a composition

comprising nanoparticles comprising at least one other chemotherapeutic agent and an albumin.

45. The method of claim 44, wherein the taxane is paclitaxel.

46. The method of claim 44, wherein the chemotherapeutic agent is rapamycin.

47. The method of claim 44, wherein the chemotherapeutic agent is 17AAG.

48. The method of claim 44, wherein the individual is human.

49. The method of claim 44, wherein the proliferative disease is cancer.

Fig.1

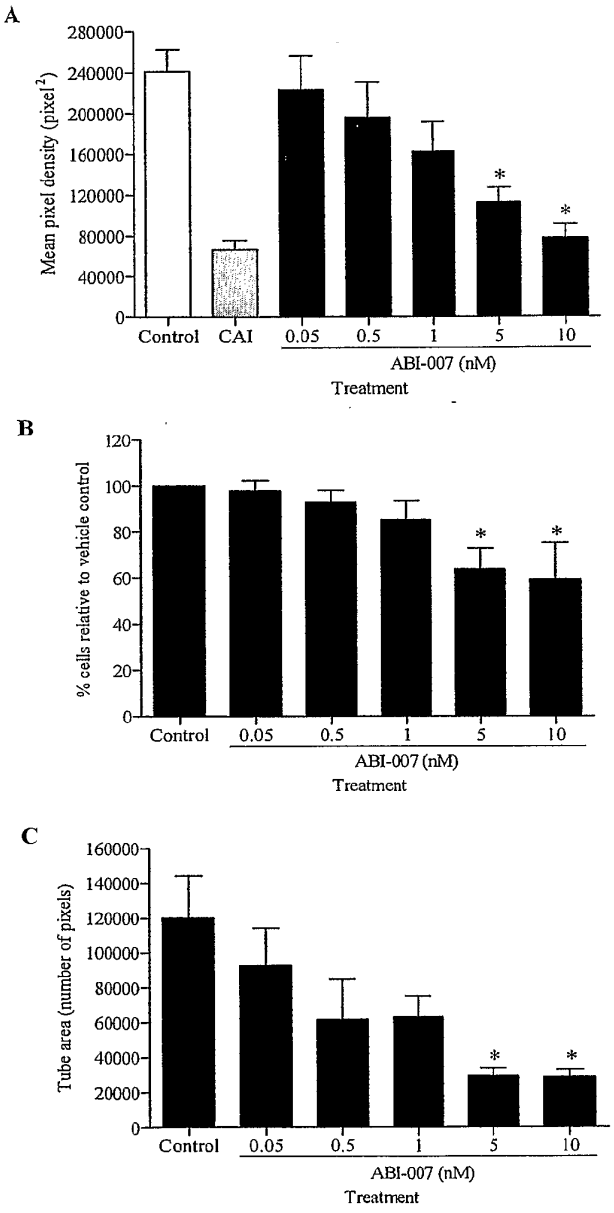


Fig. 2

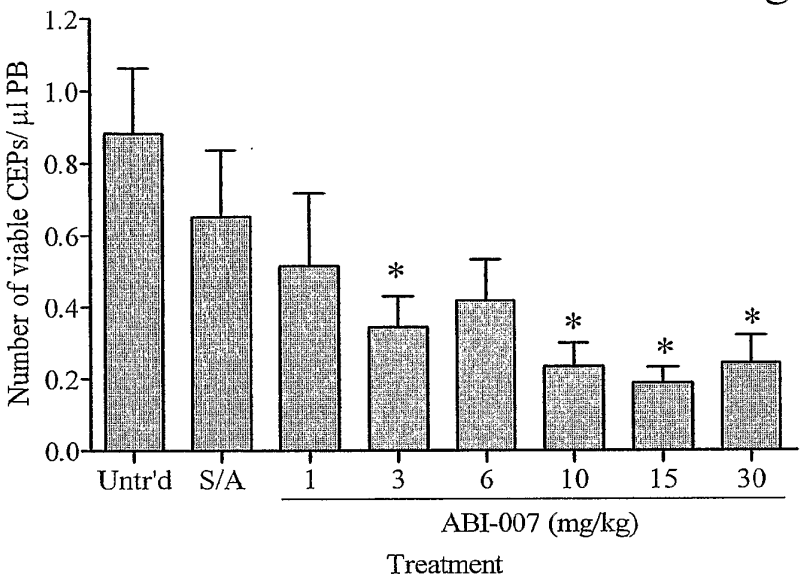


Fig. 3

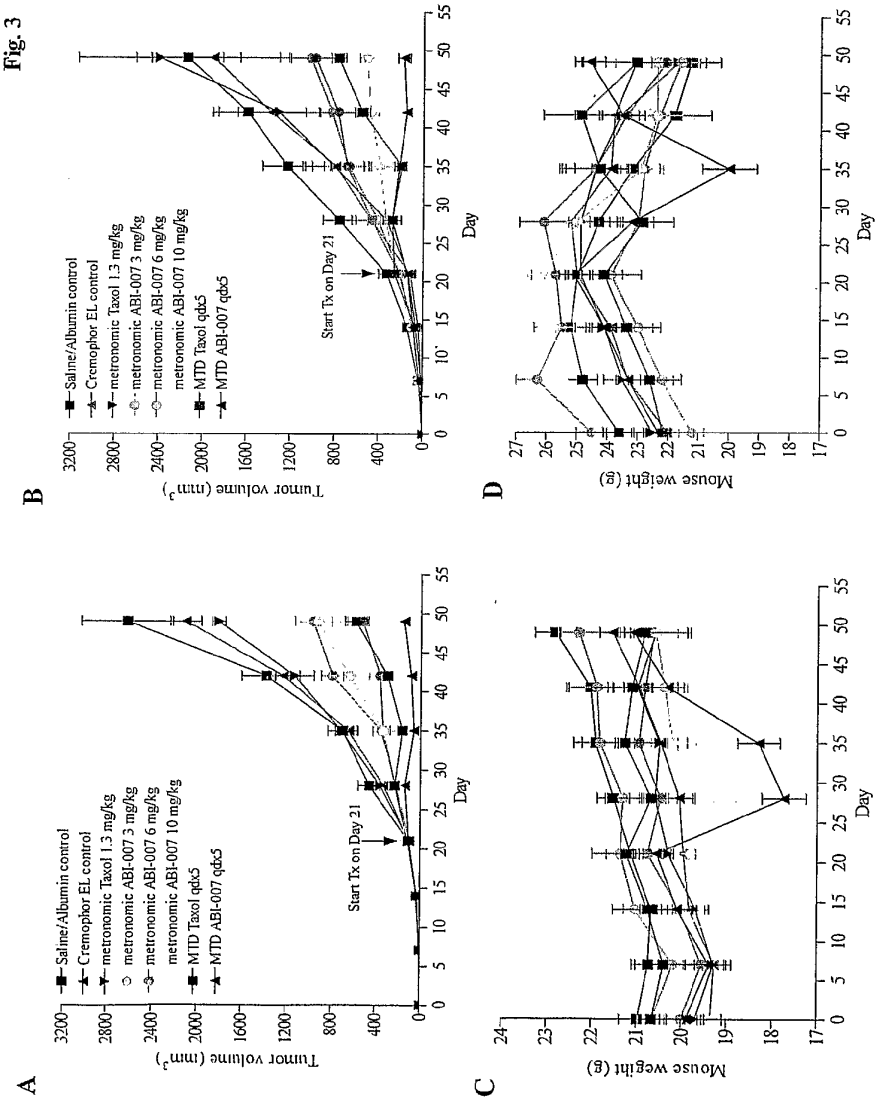


Fig. 4

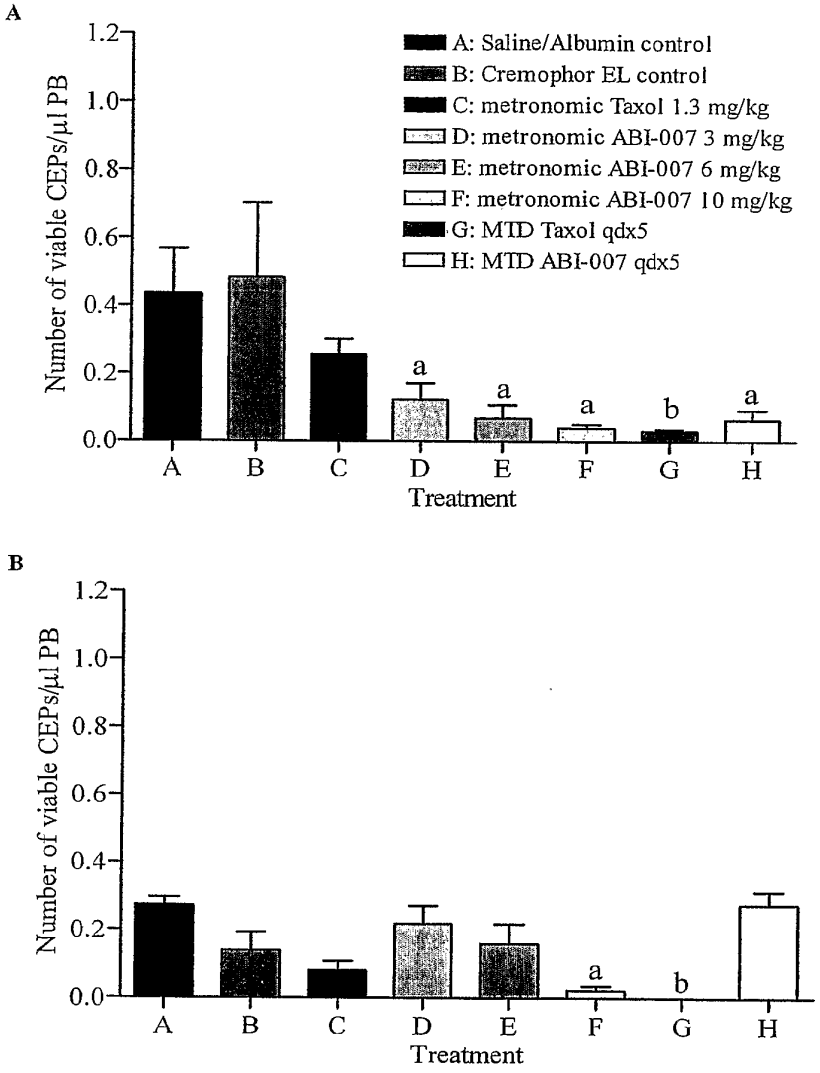


Fig. 5

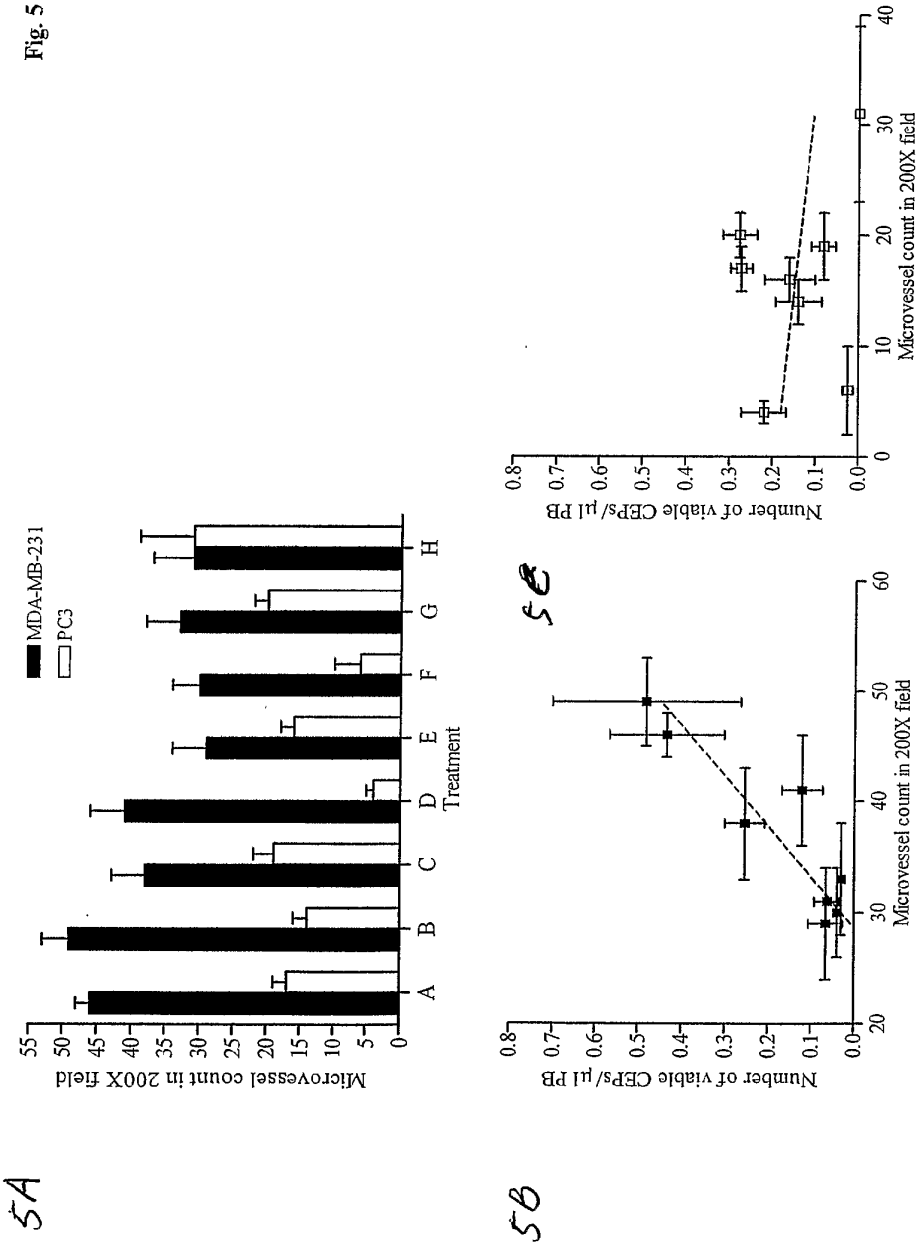


Fig. 6

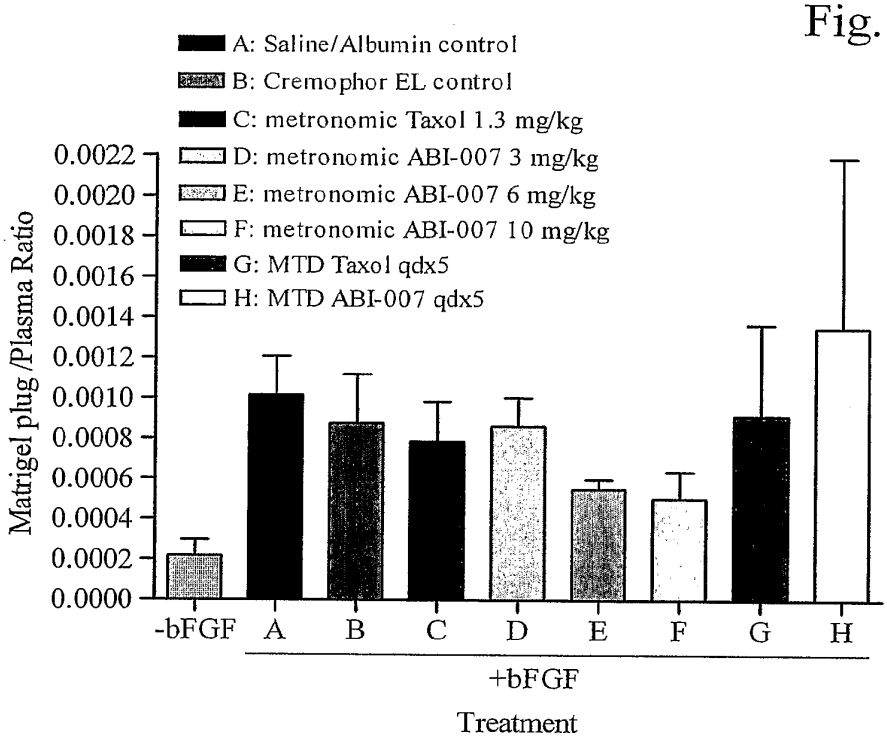
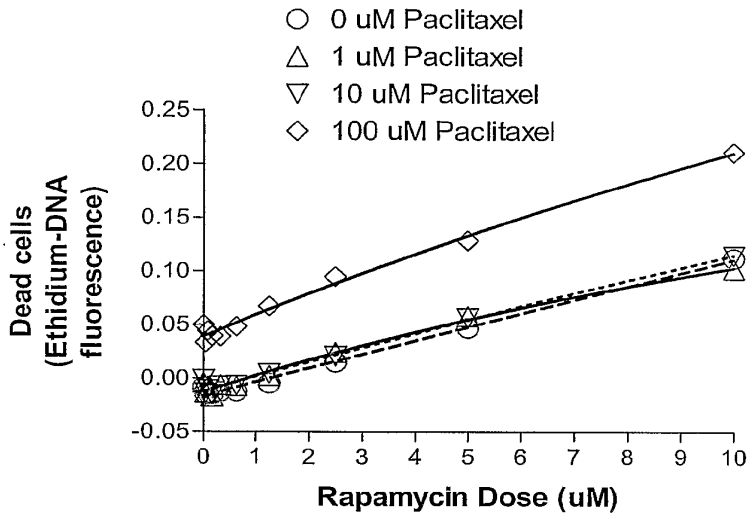


FIG. 7

A



B

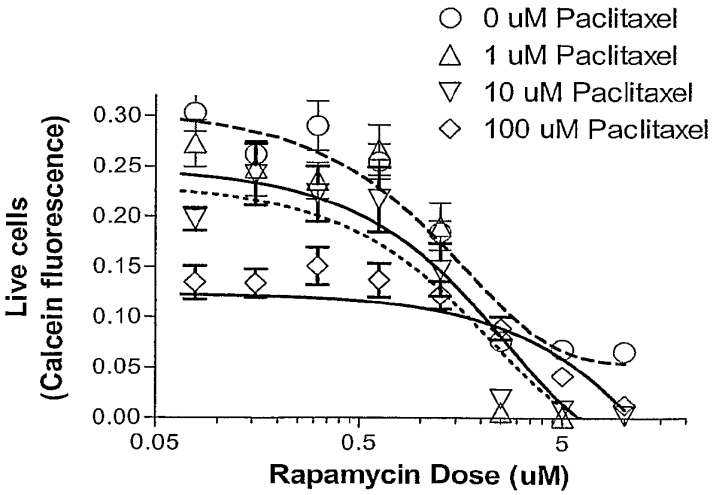


FIG. 8

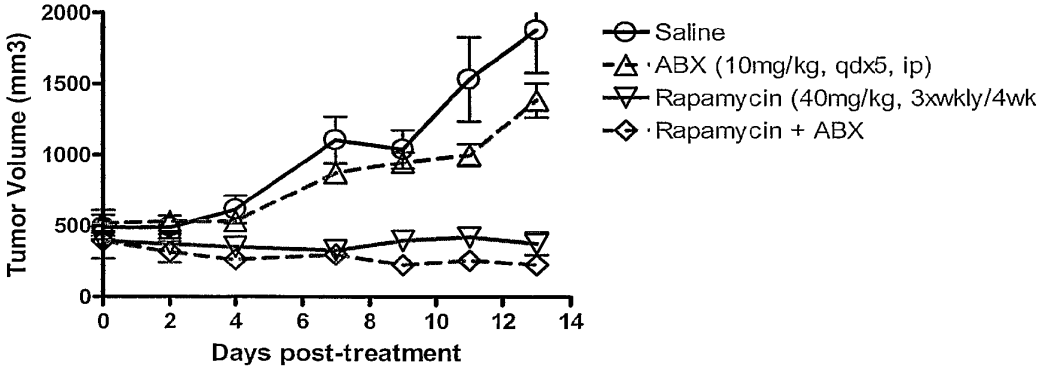
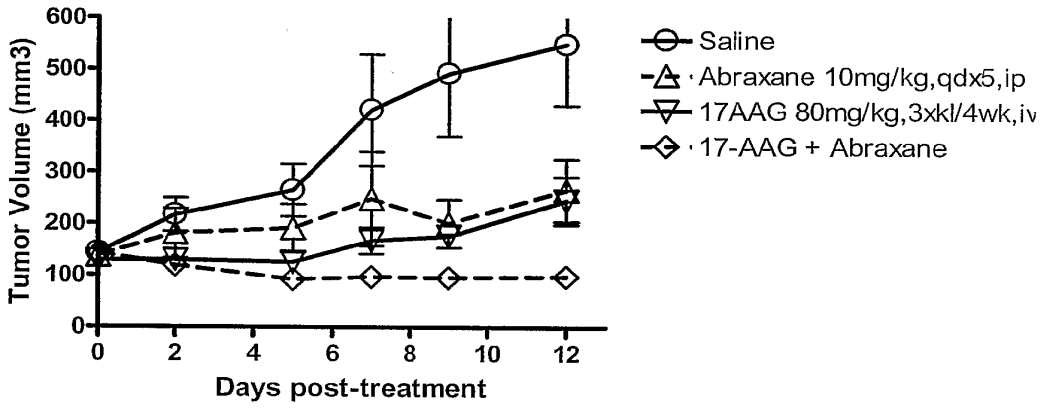


FIG. 9



INTERNATIONAL SEARCH REPORT

International application No
PCT/US2006/006167

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K31/337 A61K31/555 A61K38/22 A61K31/7068 A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, WPI Data, PAJ, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 01/89522 A (AMERICAN BIOSCIENCE, INC; DESAI, NEIL, P; SOON-SHIONG, PATRICK) 29 November 2001 (2001-11-29) examples 11,16,18	1-49
X	O'SHAUGHNESSY J A ET AL: "Weekly nanoparticle albumin paclitaxel (Abraxane) results in long-term disease control in patients with taxane-refractory metastatic breast cancer." BREAST CANCER RESEARCH AND TREATMENT, vol. 88, no. Suppl. 1, 2004, page S65, XP009068641 & 27TH ANNUAL CHARLES A COLTMAN SAN ANTONIO BREAST CANCER SYMPOSIUM; SAN ANTONIO, TX, USA; DECEMBER 08 -11, 2004 ISSN: 0167-6806 abstract	1-49

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 June 2006	07/07/2006

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Loher, F

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Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2006/006167

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	STINCHCOMBE T E ET AL: "Preliminary results of phase I trial of carboplatin (CP) in combination with ABI-007 administered weekly or every 3 weeks in patients (pts) with solid tumors." BREAST CANCER RESEARCH AND TREATMENT, vol. 94, no. Suppl. 1, 2005, page S71, XP009068639 & 28TH ANNUAL SAN ANTONIO BREAST CANCER SYMPOSIUM; SAN ANTONIO, TX, USA; DECEMBER 08 -11, 2005 ISSN: 0167-6806 abstract	1,4,5, 8-20

Form PCT/ISA/210 (continuation of second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2006/006167

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: –
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1-49 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2006/006167

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0189522 A	29-11-2001	AU 6322901 A	03-12-2001
		EP 1337249 A1	27-08-2003

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
19 November 2009 (19.11.2009)

PCT

(10) International Publication Number
WO 2009/139888 A1

(51) International Patent Classification:
A61K 9/20 (2006.01) *A61K 31/7068* (2006.01)
A61K 9/28 (2006.01)

(21) International Application Number:
PCT/US2009/002999

(22) International Filing Date:
14 May 2009 (14.05.2009)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

61/053,609	15 May 2008 (15.05.2008)	US
61/201,145	5 December 2008 (05.12.2008)	US
61/157,875	5 March 2009 (05.03.2009)	US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

WO 2009/139888 A1

(54) Title: ORAL FORMULATIONS OF CYTIDINE ANALOGS AND METHODS OF USE THEREOF

(57) Abstract: The present disclosure provides pharmaceutical compositions comprising cytidine analogs for oral administration, wherein the compositions release the cytidine analog substantially in the stomach. Also provided are methods of treating diseases and disorders using the oral formulations provided herein.

ORAL FORMULATIONS OF CYTIDINE ANALOGS AND METHODS OF USE THEREOF

I. CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Application Nos. 61/053,609, filed May 15, 2008; 61/201,145, filed December 5, 2008; and 61/157,875, filed March 5, 2009, the contents of each of which are incorporated by reference herein in their entireties.

II. FIELD

[0002] Provided herein are pharmaceutical formulations comprising cytidine analogs, or their salts, solvates, hydrates, precursors, and/or derivatives thereof, for oral administration in subjects. Also provided are methods for making the formulations and methods for using the formulations to treat diseases and disorders including cancer, disorders related to abnormal cell proliferation, hematologic disorders, and immune disorders, among others.

III. BACKGROUND

[0003] Cancer is a major worldwide public health problem; in the United States alone, approximately 570,000 cancer-related deaths were expected in 2005. *See, e.g.*, Jemal *et al.*, *CA Cancer J. Clin.* 55(1):10-30 (2005). Many types of cancer have been described in the medical literature. Examples include cancer of the blood, bone, lung (*e.g.*, non-small-cell lung cancer and small-cell lung cancer), colon, breast, prostate, ovary, brain, and intestine. The incidence of cancer continues to climb as the general population ages and as new forms of cancer develop. A continuing need exists for effective therapies to treat subjects with cancer.

[0004] Myelodysplastic syndromes (MDS) refers to a diverse group of hematopoietic stem cell disorders. MDS affects approximately 40,000-50,000 people in the U.S. and 75,000-85,000 subjects in Europe. MDS may be characterized by a cellular marrow with impaired morphology and maturation (dysmyelopoiesis), peripheral blood cytopenias, and a variable risk of progression to acute leukemia, resulting from ineffective blood cell

production. *See, e.g.*, *The Merck Manual* 953 (17th ed. 1999); List *et al.*, *J. Clin. Oncol.* 8:1424 (1990).

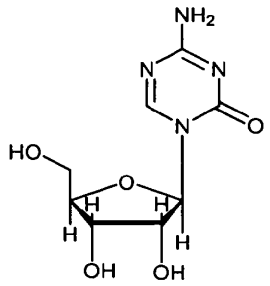
[0005] MDS are grouped together because of the presence of dysplastic changes in one or more of the hematopoietic lineages including dysplastic changes in the myeloid, erythroid, and megakaryocytic series. These changes result in cytopenias in one or more of the three lineages. Patients afflicted with MDS may develop complications related to anemia, neutropenia (infections), and/or thrombocytopenia (bleeding). From about 10% to about 70% of patients with MDS may develop acute leukemia. In the early stages of MDS, the main cause of cytopenias is increased programmed cell death (apoptosis). As the disease progresses and converts into leukemia, a proliferation of leukemic cells overwhelms the healthy marrow. The disease course differs, with some cases behaving as an indolent disease and others behaving aggressively with a very short clinical course that converts into an acute form of leukemia. The majority of people with higher risk MDS eventually experience bone marrow failure. Up to 50% of MDS patients succumb to complications, such as infection or bleeding, before progressing to AML.

[0006] Primary and secondary MDS are defined by taking into account patients' prior history: previous treatments with chemotherapy, radiotherapy or professional exposure to toxic substances are factors delineating secondary MDS (sMDS) from primary MDS. Cytogenetically, one difference between the two groups is the complexity of abnormal karyotypes; single chromosome aberrations are typical for primary MDS, while multiple changes are more frequently seen in secondary disorders. Some drugs may have specific targets such as hydroxurea for 17p and topoisomerases inhibitors for 11q23 and 21q22. The genetic changes in the malignant cells of MDS result mainly in the loss of genetic material, including probable tumor suppressor genes.

[0007] An international group of hematologists, the French-American-British (FAB) Cooperative Group, classified MDS into five subgroups, differentiating them from acute myeloid leukemia. *See, e.g.*, *The Merck Manual* 954 (17th ed. 1999); Bennett J. M., *et al.*, *Ann. Intern. Med.*, 103(4): 620-5 (1985); and Besa E. C., *Med. Clin. North Am.* 76(3): 599-617 (1992). An underlying trilineage dysplastic change in the bone marrow cells of the patients is found in all subtypes. Information is available regarding the pathobiology of MDS, certain MDS classification systems, and particular methods of treating and managing MDS. *See, e.g.*, U.S. Patent No. 7,189,740 (issued March 13, 2007), which is incorporated by reference herein in its entirety.

[0008] Nucleoside analogs have been used clinically for the treatment of viral infections and cancer. Most nucleoside analogs are classified as anti-metabolites. After they enter the cell, nucleoside analogs are successively phosphorylated to nucleoside 5'-mono-phosphates, di-phosphates, and tri-phosphates.

[0009] 5-Azacytidine (National Service Center designation NSC-102816; CAS Registry Number 320-67-2), also known as azacitidine, AZA, or 4-amino-l-β-D-ribofuranosyl-1,3,5-triazin-2(l*H*)-one, is currently marketed as the drug product VIDAZA®. 5-Azacytidine is a nucleoside analog, more specifically a cytidine analog. 5-Azacytidine is an antagonist of its related natural nucleoside, cytidine. 5-Azacytidine and 5-aza-2'-deoxycytidine (also known as decitabine, an analog of deoxycytidine) are also antagonists of deoxycytidine. A structural difference between these cytidine analogs and their related natural nucleoside is the presence of a nitrogen at position 5 of the cytosine ring in place of a carbon. 5-Azacytidine may be defined as having the molecular formula $C_8H_{12}N_4O_5$, a molecular weight of 244.21 grams per mole, and the following structure:



5-Azacytidine.

[0010] Other members of the class of cytidine analogs include, for example: l-β-D-arabinofuranosylcytosine (Cytarabine or ara-C); 5-aza-2'-deoxycytidine (Decitabine or 5-aza-CdR); pseudoisocytidine (psi ICR); 5-fluoro-2'-deoxycytidine (FCdR); 2'-deoxy-2',2'-difluorocytidine (Gemcitabine); 5-aza-2'-deoxy-2',2'-difluorocytidine; 5-aza-2'-deoxy-2'-fluorocytidine; l-β-D-ribofuranosyl-2(1*H*)-pyrimidinone (Zebularine); 2',3'-dideoxy-5-fluoro-3'-thiacytidine (Emtriva); 2'-cyclocytidine (Ancitabine); l-β-D-arabinofuranosyl-5-azacytosine (Fazarabine or ara-AC); 6-azacytidine (6-aza-CR); 5,6-dihydro-5-azacytidine (dH-aza-CR); N^4-pentyloxycarbonyl-5'-deoxy-5-fluorocytidine (Capecitabine); N^4-octadecyl-cytarabine; and elaidic acid cytarabine.

[0011] After its incorporation into replicating DNA, 5-azacytidine or 5-aza-2'-deoxycytidine forms a covalent complex with DNA methyltransferases. DNA methyltransferases are responsible for de novo DNA methylation and for reproducing established methylation patterns in daughter DNA strands of replicating DNA. Inhibition of DNA methyltransferases by 5-azacytidine or 5-aza-2'-deoxycytidine leads to DNA hypomethylation, thereby restoring normal functions to morphologically dysplastic, immature hematopoietic cells and cancer cells by re-expression of genes involved in normal cell cycle regulation, differentiation and death. The cytotoxic effects of these cytidine analogs cause the death of rapidly dividing cells, including cancer cells, that are no longer responsive to normal cell growth control mechanisms. 5-azacytidine, unlike 5-aza-2'-deoxycytidine, also incorporates into RNA. The cytotoxic effects of azacitidine may result from multiple mechanisms, including inhibition of DNA, RNAand protein synthesis, incorporation into RNA and DNA, and activation of DNA damage pathways.

[0012] 5-Azacytidine and 5-aza-2'-deoxycytidine have been tested in clinical trials and showed significant anti-tumor activity, such as, for example, in the treatment of myelodysplastic syndromes (MDS), acute myelogenous leukemia (AML), chronic myelogenous leukemia (CML), acute lymphocytic leukemia (ALL), and non Hodgkin's lymphoma (NHL). *See, e.g.*, Aparicio *et al.*, *Curr. Opin. Invest. Drugs* 3(4): 627-33 (2002). 5-Azacytidine has undergone NCI-sponsored trials for the treatment of MDS and has been approved for treating all FAB subtypes of MDS. *See, e.g.*, Kornblith *et al.*, *J. Clin. Oncol.* 20(10): 2441-2452 (2002); Silverman *et al.*, *J. Clin. Oncol.* 20(10): 2429-2440 (2002). 5-Azacytidine may alter the natural course of MDS by diminishing the transformation to AML through its cytotoxic activity and its inhibition of DNA methyltransferase. In a Phase III study, 5-azacytidine administered subcutaneously significantly prolonged survival and time to AML transformation or death in subjects with higher-risk MDS. *See, e.g.,* P. Fenaux *et al.*, *Lancet Oncol.*, 2009, 10(3):223-32; Silverman *et al.*, *Blood* 106(11): Abstract 2526 (2005).

[0013] 5-Azacytidine and other cytidine analogs are approved for subcutaneous (SC) or intravenous (IV) administration to treat various proliferative disorders. Oral dosing of cytidine analogs would be more desirable and convenient for patients and doctors, *e.g.*, by eliminating injection-site reactions that may occur with SC administration and/or by permitting improved patient compliance. However, oral delivery of cytidine analogs has proven difficult due to combinations of chemical instability, enzymatic instability, and/or poor permeability. For example, cytidine analogs have been considered acid labile and

unstable in the acidic gastric environment. Previous attempts to develop oral dosage forms of cytidine analogs have required enteric coating of the drug core to protect the active pharmaceutical ingredient (API) from what was understood and accepted to be therapeutically unacceptable hydrolysis in the stomach, such that the drug is preferably absorbed in specific regions of the lower gastrointestinal tract, such as the jejunum in the small intestine. *See, e.g.*, Sands, *et al.*, U.S. Patent Publication No. 2004/0162263 (App. No. 10/698,983). In addition, a generally accepted belief in the art has been that water leads to detrimental hydrolytic degradation of cytidine analogs during formulation, subsequently affecting the stability of the API in the dosage form. As a result, coatings applied to the drug core for prospective oral delivery of cytidine analogs have previously been limited to organic solvent-based systems to minimize exposure of the API to water.

[0014] A great need remains for oral formulations and dosage forms of cytidine analogs, such as, *e.g.*, 5-azacytidine, to potentially permit, *inter alia*, more advantageous dosing amounts or dosing periods; improved pharmacokinetic profiles, pharmacodynamic profiles, or safety profiles; evaluation of the benefits of long-term or maintenance therapies; development of improved treatment regimens that maximize biologic activity; use of cytidine analogs for treating new diseases or disorders; and/or other potential advantageous benefits.

IV. SUMMARY

[0015] Provided herein are pharmaceutical compositions comprising cytidine analogs, wherein the compositions release the API substantially in the stomach upon oral administration. Also provided are methods for making the compositions, and methods for using the compositions to treat diseases and disorders including cancer, disorders related to abnormal cell proliferation, and hematologic disorders, among others.

[0016] In certain embodiments, the cytidine analog is 5-azacytidine. In other embodiments, the cytidine analog is 5-aza-2'-deoxycytidine (decitabine or 5-aza-CdR). In yet other embodiments, the cytidine analog is, for example: 1-β-D-arabinofuranosylcytosine (Cytarabine or ara-C); pseudoisocytidine (psi ICR); 5-fluoro-2'-deoxycytidine (FCdR); 2'-deoxy-2',2'-difluorocytidine (Gemcitabine); 5-aza-2'-deoxy-2',2'-difluorocytidine; 5-aza-2'-deoxy-2'-fluorocytidine; 1-β-D-ribofuranosyl-2(1*H*)-pyrimidinone (Zebularine); 2',3'-dideoxy-5-fluoro-3'-thiacytidine (Emtriva); 2'-cyclocytidine (Ancitabine); 1-β-D-arabinofuranosyl-5-azacytosine (Fazarabine or ara-AC); 6-azacytidine (6-aza-CR); 5,6-dihydro-5-azacytidine

(dH-aza-CR); N^4-pentyloxycarbonyl-5'-deoxy-5-fluorocytidine (Capecitabine); N^4-octadecyl-cytarabine; elaidic acid cytarabine; or their derivatives or related analogs.

[0017] Certain embodiments herein provide compositions that are single unit dosage forms comprising a cytidine analog. Certain embodiments herein provide compositions that are non-enteric-coated. Certain embodiments herein provide compositions that are tablets comprising a cytidine analog. Certain embodiments herein provide compositions that are capsules comprising a cytidine analog. The capsules may be, *e.g.*, a hard gelatin capsule or a soft gelatin capsule; particular embodiments provide hydroxypropyl methylcellulose (HPMC) capsules. In certain embodiments, the single unit dosage forms optionally further contain one or more excipients. In certain embodiments, the tablets optionally further contain one or more excipients. In other embodiments, the capsules optionally further contain one or more excipients. In certain embodiments, the composition is a tablet that effects an immediate release of the API upon oral administration. In other embodiments, the composition is a tablet that effects a controlled release of the API substantially in the stomach. In certain embodiments, the composition is a capsule that effects an immediate release of the API upon oral administration. In other embodiments, the composition is a capsule that effects a controlled release of the API substantially in the stomach. In particular embodiments, the tablet contains a drug core that comprises a cytidine analog, and optionally further contains a coating of the drug core, wherein the coating is applied to the drug core using an aqueous solvent, such as, for example, water, or non-aqueous solvent, such as, for example ethanol.

[0018] Certain embodiments herein provide methods of making formulations of cytidine analogs intended for oral delivery. Further provided are articles of manufacture containing packaging material, an oral formulation of a cytidine analog, and a label that indicates that the formulation is for the treatment of certain diseases or disorders including, *e.g.*, a cancer, a disorder related to abnormal cell proliferation, a hematologic disorder, or an immune disorder.

[0019] Certain embodiments herein provide methods of using the formulations provided herein to treat diseases or disorders including, *e.g.*, cancer, disorders related to abnormal cell proliferation, hematologic disorders, or immune disorders, among others. In certain embodiments, the formulations of cytidine analogs are orally administered to subjects in need thereof to treat a cancer or a hematological disorder, such as, for example, MDS, AML, ALL, CML, NHL, leukemia, or lymphoma; or a solid tumor, such as, for example, sarcoma, melanoma, carcinoma, or cancer of the colon, breast, ovary, gastrointestinal system, kidney,

lung (*e.g.*, non-small-cell lung cancer and small-cell lung cancer), testicle, prostate, pancreas or bone. In certain embodiments, the formulations of cytidine analogs are orally administered to subjects in need thereof to treat an immune disorder. In certain embodiments, the oral formulations provided herein are co-administered with one or more therapeutic agents to provide a synergistic therapeutic effect in subjects in need thereof. In certain embodiments, the oral formulations provided herein are co-administered with one or more therapeutic agents to provide a resensitization effect in subjects in need thereof. The co-administered agents may be a cancer therapeutic agent, as described herein. In certain embodiments, the co-administered agent(s) may be dosed, *e.g.*, orally or by injection.

[0020] In particular embodiments, provided herein are tablets containing 5-azacytidine and methods for making and using the tablets to treat cancer, disorders related to abnormal cell proliferation, or hematologic disorders. In certain embodiments, the tablets optionally further contain one or more excipients such as, for example, glidants, diluents, lubricants, colorants, disintegrants, granulating agents, binding agents, polymers, and/or coating agents. Examples of ingredients useful in preparing certain formulations provided herein are described in, *e.g.*, Etter *et al.*, U.S. Patent Application Publication No. 2008/0057086 (App. No. 11/849,958), which is incorporated herein by reference in its entirety.

[0021] Specific embodiments herein provide, *inter alia*, pharmaceutical compositions comprising a therapeutically effective amount of 5-azacytidine, wherein the composition releases the 5-azacytidine substantially in the stomach following oral administration to a subject. Further embodiments provide the aforementioned compositions, which: are immediate release compositions; do not have an enteric coating (*i.e.*, are non-enteric-coated); are tablets; are capsules; further comprise an excipient selected from any excipient disclosed herein; further comprise a permeation enhancer; further comprise d-alpha-tocopheryl polyethylene glycol 1000 succinate; further comprise a permeation enhancer in the formulation at about 2% by weight relative to the total weight of the formulation; are essentially free of a cytidine deaminase inhibitor; are essentially free of tetrahydrouridine; have an amount of 5-azacytidine of at least about 40 mg; have an amount of 5-azacytidine of at least about 400 mg; have an amount of 5-azacytidine of at least about 1000 mg; achieve an area-under-the-curve value of at least about 200 ng-hr/mL following oral administration to a subject; achieve an area-under-the-curve value of at least about 400 ng-hr/mL following oral administration to a subject; achieve a maximum plasma concentration of at least about 100 ng/mL following oral administration to a subject; achieve a maximum plasma concentration

of at least about 200 ng/mL following oral administration to a subject; achieve a time to maximum plasma concentration of less than about 90 minutes following oral administration to a subject; and/or achieve a time to maximum plasma concentration of less than about 60 minutes following oral administration to a subject.

[0022] Specific embodiments herein provide a pharmaceutical composition for oral administration comprising a therapeutically effective amount of 5-azacytidine, which releases the 5-azacytidine substantially in the stomach and achieves an area-under-the-curve value of at least about 200 ng-hr/mL following oral administration.

[0023] Specific embodiments herein provide a pharmaceutical composition for oral administration comprising a therapeutically effective amount of 5-azacytidine, which releases the 5-azacytidine substantially in the stomach and achieves an area-under-the-curve value of at least about 400 ng-hr/mL following oral administration.

[0024] Specific embodiments herein provide a pharmaceutical composition for oral administration comprising a therapeutically effective amount of 5-azacytidine, which releases the 5-azacytidine substantially in the stomach and achieves a maximum plasma concentration of at least about 100 ng/mL following oral administration.

[0025] Specific embodiments herein provide a pharmaceutical composition for oral administration comprising a therapeutically effective amount of 5-azacytidine, which releases the 5-azacytidine substantially in the stomach and achieves a maximum plasma concentration of at least about 200 ng/mL following oral administration.

[0026] Specific embodiments herein provide a pharmaceutical composition for oral administration comprising a therapeutically effective amount of 5-azacytidine, which releases the 5-azacytidine substantially in the stomach and achieves a time to maximum plasma concentration of, *e.g.*, less than about 6 hr, less than about 5 hr, less than about 4 hr, less than about 3 hr, less than about 2.5 hr, less than about 2 hr, less than about 1.5 hr, less than about 1 hr, less than about 45 min, or less than about 30 min following oral administration. In specific embodiments, the presence of food may affect (*e.g.*, extend) the total exposure and/or time to maximum plasma concentration.

[0027] Specific embodiments herein provide a pharmaceutical composition for oral administration comprising a therapeutically effective amount of 5-azacytidine, which releases the 5-azacytidine substantially in the stomach and achieves a time to maximum plasma concentration of less than about 60 minutes following oral administration.

[0028] Specific embodiments herein provide any of the aforementioned compositions, as single unit dosage forms, tablets, or capsules.

[0029] Specific embodiments herein provide, *inter alia*, methods for treating a subject having a disease associated with abnormal cell proliferation, comprising orally administering to the subject a pharmaceutical composition comprising a therapeutically effective amount of 5-azacytidine, wherein the composition releases the 5-azacytidine substantially in the stomach following oral administration to the subject. Further embodiments herein provide the aforementioned methods, in which: the disease is myelodysplastic syndrome; the disease is acute myelogenous leukemia; the method further comprises co-administering to the subject in need thereof an additional therapeutic agent selected from any additional therapeutic agent disclosed herein; the composition is an immediate release composition; the composition does not have an enteric coating; the composition further comprises a permeation enhancer; the composition further comprises the permeation enhancer d-alpha-tocopheryl polyethylene glycol 1000 succinate; the composition further comprises d-alpha-tocopheryl polyethylene glycol 1000 succinate in the formulation at about 2% by weight relative to the total weight of the formulation; the method further comprises not co-administering a cytidine deaminase inhibitor with the cytidine analog; the composition is a single unit dosage form; the composition is a tablet; the composition is a capsule; the composition further comprises an excipient selected from any excipient disclosed herein; the amount of 5-azacytidine is at least about 40 mg; the amount of 5-azacytidine is at least about 400 mg; the amount of 5-azacytidine is at least about 1000 mg; the method achieves an area-under-the-curve value of at least about 200 ng-hr/mL following oral administration to the subject; the method achieves an area-under-the-curve value of at least about 400 ng-hr/mL following oral administration to the subject; the method achieves a maximum plasma concentration of at least about 100 ng/mL following oral administration to the subject; the method achieves a maximum plasma concentration of at least about 200 ng/mL following oral administration to the subject; the method achieves a time to maximum plasma concentration of less than about 90 minutes following oral administration to the subject; and/or the method achieves a time to maximum plasma concentration of less than about 60 minutes following oral administration to the subject

[0030] Specific embodiments herein provide, *inter alia*, pharmaceutical compositions comprising a therapeutically effective amount of 5-azacytidine, wherein the compositions are for treating a disease or disorder associated with abnormal cell proliferation, wherein the

compositions are prepared for oral administration, and wherein the compositions are prepared for release of the 5-azacytidine substantially in the stomach. Further embodiments herein provide the aforementioned compositions, which: have an amount of 5-azacytidine of about 40 mg, about 400 mg, or about 1000 mg; are prepared to achieve an area-under-the-curve value of at least about 200 ng-hr/mL or 400 ng-hr/mL following oral administration; are prepared to achieve a maximum plasma concentration of at least about 100 ng/mL or 200 ng/mL following oral administration; are prepared to achieve a time to maximum plasma concentration of less than about 60 minutes or 90 minutes after being administered; are prepared in the form of an immediate release composition; are prepared for oral administration in combination with an additional therapeutic agent selected from any additional therapeutic agent disclosed herein; are for treating myelodysplastic syndrome or acute myelogenous leukemia; further comprise a permeation enhancer; which further comprise the permeation enhancer d-alpha-tocopheryl polyethylene glycol 1000 succinate; are single unit dosage forms; are tablets or capsules; and/or further comprise an excipient selected from any excipient disclosed herein.

[0031] Specific embodiments herein provide, *inter alia*, uses of 5-azacytidine for the preparation of a pharmaceutical composition for treating a disease associated with abnormal cell proliferation, wherein the composition is prepared for oral administration, and wherein the composition is prepared for release of the 5-azacytidine substantially in the stomach. Further embodiments herein provide the aforementioned uses, in which: the disease is myelodysplastic syndrome or acute myelogenous leukemia; the amount of 5-azacytidine is selected from any amount disclosed herein; and/or the composition is prepared for immediate release. Further embodiments provide, *inter alia*, methods for treating a subject having a disease or disorder provided herein by administering a pharmaceutical compositions provided herein, wherein the treatment results in improved survival of the subject.

V. BRIEF DESCRIPTION OF THE DRAWINGS

[0032] Figure 1 represents processes and steps that may be used to make particular tablets comprising azacitidine for oral dosing; in specific embodiments, one or more steps may be optionally omitted.

[0033] Figure 2 represents human PK profiles following 75 mg/m^2 SC dosing of azacitidine on Days 1 and 7 in a multiple dose escalation study (n = 18). The X-axis represents time; the Y-axis represents azacitidine plasma concentrations (mean $\pm$ SD).

[0034] Figure 3 represents human PK profiles following SC (75 mg/m^2) and PO (240 mg, 300 mg, and 360 mg) dosing of azacitidine in a multiple dose escalation study. The azacitidine plasma PK profiles are compared among various doses. The X-axis represents time; the Y-axis represents azacitidine plasma concentrations (mean $\pm$ SD).

[0035] Figure 4 represents PD data from an individual patient (Subject 02008, 80 year old male, RAEB-1) collected during a multiple dose escalation study. The patient was dosed with azacitidine Formulation #3, 240 mg. Platelets (K/μL), Hgb (g/dL), ANC (K/μL), and Relative BM Blast (%) are plotted versus sampling dates over the course of the study.

[0036] Figure 5 represents PD data from an individual patient (Subject 02007, 76 year old male, CMML) collected during a multiple dose escalation study. The patient was dosed with azacitidine Formulation #3, 240 mg. Platelets (K/μL), Hgb (g/dL), ANC (K/μL), and Relative BM Blast (%) are plotted versus sampling dates over the course of the study.

[0037] Figure 6 represents PD data from an individual patient (Subject 02004, 61 year old male, MDS, MDACC) collected during a multiple dose escalation study. The patient was dosed with azacitidine Formulation 1, 120 mg. Platelets (K/μL), Hgb (g/dL), ANC (K/μL), and Relative BM Blast (%) are plotted versus sampling dates over the course of the study.

[0038] Figure 7 represents a study design of a Rapid Aza Clinical Evaluation (RACE) study CL008. Doses given on various days within a treatment cycle are depicted. Dose may be administered $\pm$ 1 day, as long as there is at least 48 hours between doses.

[0039] Figure 8 represents azacitidine human PK profiles from an individual patient (Subject 106003, N = 1) following SC (124 mg, 75 mg/m^2) and PO (180 mg, 360 mg, 1,200 mg, Formulation 4) dosing of azacitidine from a RACE clinical study. AUC(0-t) values for the SC and PO doses are depicted.

[0040] Figure 9 represents azacitidine human PK profiles from an individual patient (Subject 106004, N = 1) following SC (120 mg, 75 mg/m^2) and PO (180 mg, 360 mg, 1,200 mg, Formulation 6) dosing of azacitidine from a RACE clinical study. AUC(0-∞) values for the SC and PO doses are depicted.

[0041] Figure 10 represents human PK profiles (linear scale) following SC and oral administration of azacitidine in clinical studies.

[0042] Figure 11 represents human PK profiles (semi-log scale) following SC and oral administration of azacitidine in clinical studies.

[0043] Figure 12 represents human AUC values following SC dosing of azacitidine and oral dosing of azacitidine with Formulations #3, #4, and #6 at various dosage levels in

clinical studies (CL005 and CL008).

[0044] Figure 13 represents human Cmax values in patients following SC dosing of azacitidine and oral dosing of azacitidine with Formulations #3, #4, and #6 at various dosage levels in clinical studies.

[0045] Figure 14 represents relative oral bioavailability in humans following oral dosing of azacitidine with Formulations #3, #4, and #6 at various dosage levels.

[0046] Figure 15 represents percent exposure in humans relative to SC administration following oral dosing of azacitidine with Formulations #3, #4, and #6 at various dosage levels.

[0047] Figure 16 represents profiles of human plasma concentration versus time (linear scale) following oral dosing of azacitidine with Formulations #3 and #6 and 180 mg (n=6).

[0048] Figure 17 represents linear scale profiles of human plasma concentration (ng/ml) versus time (hr) following oral dosing of azacitidine with Formulations #3 and #6 and 360 mg (n=6).

[0049] Figure 18 represents a plot of values for individual ("ind") and mean azacitidine ACU(0-inf) (ng*hr/ml) versus azacitidine dose (mg), with calculated linear regression lines for Formulations #3 and #6.

[0050] Figure 19 represents a comparison of azacitidine relative oral bioavailability (%) (mean ± SD) versus azacitidine dose (mg) following dosing with Formulation #3 or #6.

[0051] Figure 20 represents a comparison of azacitidine exposure as compared to SC dose (mean ± SD) versus azacitidine dose (mg) following oral administration of Formulation #3 or #6.

VI. DETAILED DESCRIPTION

[0052] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art. All publications and patents referred to herein are incorporated by reference herein in their entireties.

A. Definitions

[0053] As used in the specification and the accompanying claims, the indefinite articles "a" and "an" and the definite article "the" include plural as well as singular referents, unless the context clearly dictates otherwise.

[0054] The term "about" or "approximately" means an acceptable error for a particular value as determined by one of ordinary skill in the art, which depends in part on how the

value is measured or determined. In certain embodiments, the term "about" or "approximately" means within 1, 2, 3, or 4 standard deviations. In certain embodiments, the term "about" or "approximately" means within 30%, 25%, 20%, 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.1%, or 0.05% of a given value or range.

[0055] As used herein, and unless otherwise specified, the terms "treat," "treating" and "treatment" refer to the eradication or amelioration of a disease or disorder, or of one or more symptoms associated with the disease or disorder. In certain embodiments, the terms refer to minimizing the spread or worsening of the disease or disorder resulting from the administration of one or more prophylactic or therapeutic agents to a subject with such a disease or disorder. In some embodiments, the terms refer to the administration of a compound or dosage form provided herein, with or without one or more additional active agent(s), after the onset of symptoms of the particular disease.

[0056] As used herein, and unless otherwise specified, the terms "prevent," "preventing" and "prevention" refer to the prevention of the onset, recurrence or spread of a disease or disorder, or of one or more symptoms thereof. In certain embodiments, the terms refer to the treatment with or administration of a compound or dosage form provided herein, with or without one or more other additional active agent(s), prior to the onset of symptoms, particularly to subjects at risk of disease or disorders provided herein. The terms encompass the inhibition or reduction of a symptom of the particular disease. Subjects with familial history of a disease in particular are candidates for preventive regimens in certain embodiments. In addition, subjects who have a history of recurring symptoms are also potential candidates for prevention. In this regard, the term "prevention" may be interchangeably used with the term "prophylactic treatment."

[0057] As used herein, and unless otherwise specified, the terms "manage," "managing" and "management" refer to preventing or slowing the progression, spread or worsening of a disease or disorder, or of one or more symptoms thereof. Often, the beneficial effects that a subject derives from a prophylactic and/or therapeutic agent do not result in a cure of the disease or disorder. In this regard, the term "managing" encompasses treating a subject who had suffered from the particular disease in an attempt to prevent or minimize the recurrence of the disease.

[0058] As used herein, amelioration of the symptoms of a particular disorder by administration of a particular pharmaceutical composition refers to any lessening, whether

permanent or temporary, lasting or transient, that can be attributed to or associated with administration of the composition.

[0059] As used herein, and unless otherwise specified, the terms "therapeutically effective amount" and "effective amount" of a compound mean an amount sufficient to provide a therapeutic benefit in the treatment or management of a disease or disorder, or to delay or minimize one or more symptoms associated with the disease or disorder. A "therapeutically effective amount" and "effective amount" of a compound mean an amount of therapeutic agent, alone or in combination with one or more other agent(s), which provides a therapeutic benefit in the treatment or management of the disease or disorder. The terms "therapeutically effective amount" and "effective amount" can encompass an amount that improves overall therapy, reduces or avoids symptoms or causes of disease or disorder, or enhances the therapeutic efficacy of another therapeutic agent.

[0060] As used herein, and unless otherwise specified, a "prophylactically effective amount" of a compound is an amount sufficient to prevent a disease or disorder, or prevent its recurrence. A prophylactically effective amount of a compound means an amount of therapeutic agent, alone or in combination with one or more other agent(s), which provides a prophylactic benefit in the prevention of the disease. The term "prophylactically effective amount" can encompass an amount that improves overall prophylaxis or enhances the prophylactic efficacy of another prophylactic agent.

[0061] "Tumor," as used herein, refers to all neoplastic cell growth and proliferation, whether malignant or benign, and all pre-cancerous and cancerous cells and tissues. "Neoplastic," as used herein, refers to any form of dysregulated or unregulated cell growth, whether malignant or benign, resulting in abnormal tissue growth. Thus, "neoplastic cells" include malignant and benign cells having dysregulated or unregulated cell growth.

[0062] The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include, but are not limited to blood borne (*e.g.*, lymphoma, leukemia) and solid tumors.

[0063] The terms "composition," "formulation," and "dosage form," as used herein are intended to encompass compositions comprising the specified ingredient(s) (in the specified amounts, if indicated), as well as any product(s) which result, directly or indirectly, from combination of the specified ingredient(s) in the specified amount(s). By "pharmaceutical" or "pharmaceutically acceptable" it is meant that any diluent(s), excipient(s) or carrier(s) in

the composition, formulation, or dosage form are compatible with the other ingredient(s) and not deleterious to the recipient thereof. Unless indicated otherwise, the terms "composition," "formulation," and "dosage form" are used herein interchangeably.

[0064] The term "immediate release," when used herein in reference to a composition, formulation, or dosage form provided herein, means that the composition, formulation, or dosage form does not comprise a component (*e.g.*, a coating) that serves to delay the spatial and/or temporal release of some or all of the API from the composition, formulation, or dosage form beyond the stomach following oral administration. In certain embodiments, an immediate release composition, formulation, or dosage form is one that releases the API substantially in the stomach following oral administration. In specific embodiments, an immediate release composition, formulation, or dosage form is one that is not delayed-release. In specific embodiments, an immediate release composition, formulation, or dosage form is one that does not comprise an enteric coating.

[0065] The term "non-enteric-coated," when used herein, refers to a pharmaceutical composition, formulation, or dosage form that does not comprise a coating intended to release the active ingredient(s) beyond the stomach (*e.g.*, in the intestine). In certain embodiments, a non-enteric-coated composition, formulation, or dosage form is designed to release the active ingredient(s) substantially in the stomach.

[0066] The term "substantially in the stomach," when used herein in reference to a composition, formulation, or dosage form provided herein, means that at least about 99%, at least about 95%, at least about 90%, at least about 85%, at least about 80%, at least about 75%, at least about 70%, at least about 65%, at least about 60%, at least about 55%, at least about 50%, at least about 45%, at least about 40%, at least about 35%, at least about 30%, at least about 25%, at least about 20%, at least about 15%, or at least about 10% of the cytidine analog is released in the stomach. The term "released in the stomach" and related terms as used herein refer to the process whereby the cytidine analog is made available for uptake by or transport across cells lining the stomach and then made available to the body.

[0067] The term "subject" is defined herein to include animals such as mammals, including, but not limited to, primates (*e.g.*, humans), cows, sheep, goats, horses, dogs, cats, rabbits, rats, mice and the like. In specific embodiments, the subject is a human.

[0068] The terms "co-administration" and "in combination with" include the administration of two or more therapeutic agents either simultaneously, concurrently or sequentially within no specific time limits. In one embodiment, the agents are present in the

cell or in the subject's body at the same time or exert their biological or therapeutic effect at the same time. In one embodiment, the therapeutic agents are in the same composition or unit dosage form. In other embodiments, the therapeutic agents are in separate compositions or unit dosage forms. In certain embodiments, a first agent can be administered prior to (*e.g.*, 5 minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, or 12 weeks before), concomitantly with, or subsequent to (*e.g.*, 5 minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, or 12 weeks after) the administration of a second therapeutic agent.

[0069] The term "isotopic composition" refers to the amount of each isotope present in a given atomic position, and "natural isotopic composition" refers to the naturally occurring isotopic composition or abundance for a given atomic position. Atomic positions containing their natural isotopic composition may also be referred to herein as "non-enriched." Unless otherwise designated, the atomic positions of the compounds recited herein are meant to represent any stable isotope of that atom. For example, unless otherwise stated, when a position is designated specifically as "H" or "hydrogen," the position is understood to have hydrogen at its natural isotopic composition.

[0070] The term "isotopically enriched" refers to an atomic position having an isotopic composition other than the natural isotopic composition of that atom. "Isotopically enriched" may also refer to a compound containing at least one atomic position having an isotopic composition other than the natural isotopic composition of that atom. As used herein, an "isotopologue" is an isotopically enriched compound.

[0071] The term "isotopic enrichment" refers to the percentage of incorporation of an amount of a specific isotope at a given atomic position in a molecule in the place of that atom's natural isotopic composition. For example, deuterium enrichment of 1% at a given position means that 1% of the molecules in a given sample contain deuterium at the specified position. Because the naturally occurring distribution of deuterium is about 0.0156%, deuterium enrichment at any position in a compound synthesized using non-enriched starting materials is about 0.0156%.

[0072] The term "isotopic enrichment factor" refers to the ratio between the isotopic composition and the natural isotopic composition of a specified isotope.

[0073] With regard to the compounds provided herein, when a particular atomic position is designated as having deuterium or "D," it is understood that the abundance of deuterium at that position is substantially greater than the natural abundance of deuterium, which is about 0.015%. A position designated as having deuterium typically has a minimum isotopic enrichment factor of, in particular embodiments, at least 1000 (15% deuterium incorporation), at least 2000 (30% deuterium incorporation), at least 3000 (45% deuterium incorporation), at least 3500 (52.5% deuterium incorporation), at least 4000 (60% deuterium incorporation), at least 4500 (67.5% deuterium incorporation), at least 5000 (75% deuterium incorporation), at least 5500 (82.5% deuterium incorporation), at least 6000 (90% deuterium incorporation), at least 6333.3 (95% deuterium incorporation), at least 6466.7 (97% deuterium incorporation), at least 6600 (99% deuterium incorporation), or at least 6633.3 (99.5% deuterium incorporation) at each designated deuterium position.

[0074] The isotopic enrichment and isotopic enrichment factor of the compounds provided herein can be determined using conventional analytical methods known to one of ordinary skill in the art, including, *e.g.*, mass spectrometry, nuclear magnetic resonance spectroscopy, and crystallography.

B. Cytidine Analogs

1. Overview

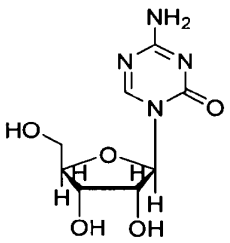
[0075] Provided herein are dosage forms, pharmaceutical formulations and compositions comprising cytidine analogs that release the API substantially in the stomach upon oral administration. In certain embodiments, the cytidine analog is 5-azacytidine. In certain embodiments, the cytidine analog is 5-aza-2'-deoxycytidine (decitabine or 5-aza-CdR). In certain embodiments, the cytidine analog is, for example: l-β-D-arabinofuranosylcytosine (Cytarabine or ara-C); pseudoiso-cytidine (psi ICR); 5-fluoro-2'-deoxycytidine (FCdR); 2'-deoxy-2',2'-difluorocytidine (Gemcitabine); 5-aza-2'-deoxy-2',2'-difluorocytidine; 5-aza-2'-deoxy-2'-fluorocytidine; l-β-D-ribofuranosyl-2(1*H*)-pyrimidinone (Zebularine); 2',3'-dideoxy-5-fluoro-3'-thiacytidine (Emtriva); 2'-cyclocytidine (Ancitabine); l-β-D-arabinofuranosyl-5-azacytosine (Fazarabine or ara-AC); 6-azacytidine (6-aza-CR); 5,6-dihydro-5-azacytidine (dH-aza-CR); N^4-pentyloxy-carbonyl-5'-deoxy-5-fluorocytidine (Capecitabine); N^4-octadecyl-cytarabine; elaidic acid cytarabine; or a conjugated compound comprising a cytidine analog and a fatty acid (*e.g.*, an azacitidine–fatty acid conjugate, including, but not limited to, CP-4200 (Clavis Pharma ASA) or a compound disclosed in WO 2009/042767, such as aza-C-5'-petroselinic acid ester or aza-C-5'-petroselaidic acid ester).

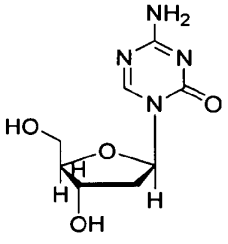
[0076] In certain embodiments, cytidine analogs provided herein include esterified derivatives of cytidine analogs, such as, *e.g.*, esterified derivatives of 5-azacytidine. In particular embodiments, esterified derivatives are cytidine analogs that contain an ester moiety (*e.g.*, an acetyl group) at one or more positions on the cytidine analog molecule. Esterified derivatives may be prepared by any method known in the art. In certain embodiments, esterified derivatives of a cytidine analog serve as prodrugs of the cytidine analog, such that, *e.g.*, following administration of an esterified derivative, the derivative is deacetylated *in vivo* to yield the cytidine analog. A particular embodiment herein provides 2′,3′,5′-triacetyl-5-azacytidine (TAC), which possesses favorable physical-chemical and therapeutic properties. *See, e.g.*, International Publication No. WO 2008/092127 (International Application No. PCT/US2008/052124); Ziemba, A.J., *et al.*, "Development of Oral Demethylating Agents for the Treatment of Myelodysplastic Syndrome" (Abstract No. 3369), In: *Proceedings of the 100th Annual Meeting of the American Association for Cancer Research*; 2009 Apr. 18-22; Denver, Co. Philadelphia (PA): AACR; 2009 (both of which are incorporated by reference herein in their entireties).

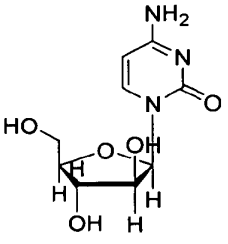
[0077] In certain embodiments, the cytidine analogs provided herein include any compound which is structurally related to cytidine or deoxycytidine and functionally mimics and/or antagonizes the action of cytidine or deoxycytidine. Certain embodiments herein provide salts, cocrystals, solvates (*e.g.*, hydrates), complexes, prodrugs, precursors, metabolites, and/or other derivatives of the cytidine analogs provided herein. For example, particular embodiments provide salts, cocrystals, solvates (*e.g.*, hydrates), complexes, precursors, metabolites, and/or other derivatives of 5-azacytidine. Certain embodiments provide cytidine analogs that are not salts, cocrystals, solvates (*e.g.*, hydrates), or complexes of the cytidine analogs provided herein. For example, particular embodiments provide 5-azacytidine in a non-ionized, non-solvated (*e.g.*, anhydrous), non-complexed form. Certain embodiments herein provide mixtures of two or more cytidine analogs provided herein.

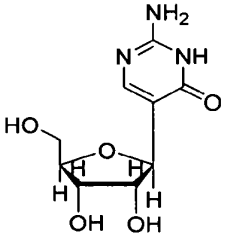
[0078] Cytidine analogs provided herein may be prepared using synthetic methods and procedures referenced herein or otherwise available in the literature. For example, particular methods for synthesizing 5-azacytidine are taught in, *e.g.*, U.S. Patent No. 7,038,038 and references discussed therein, each of which is incorporated herein by reference. 5-Azacytidine is also available from Celgene Corporation, Warren, NJ. Other cytidine analogs provided herein may be prepared using previously disclosed synthetic procedures available to a person of ordinary skill in the art.

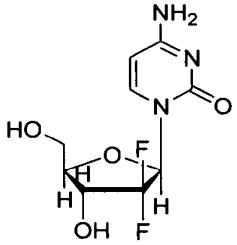
[0079] In certain embodiments, exemplary cytidine analogs have the structures provided below:

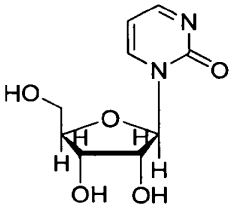

Azacitidine

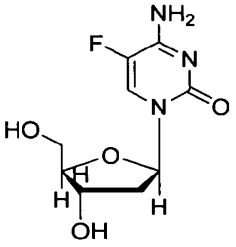

Decitabine

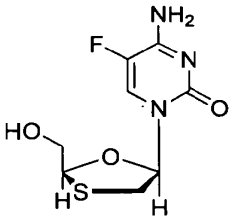

Cytarabine (Ara-C)

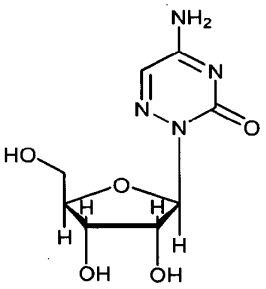

Pseudoisocytidine (psi ICR)

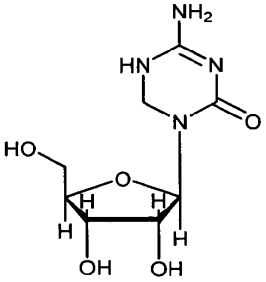

Gemcitabine


Zebularine


FCdR


Emtriva


6-Azacytidine


5-6-Dihydro-5-azacytidine

2. Isotopically Enriched Cytidine Analogs

[0080] Particular embodiments herein provide isotopically enriched cytidine analogs, prodrugs thereof, synthetic intermediates thereof, and metabolites thereof. For example, specific embodiments herein provide isotopically enriched 5-azacytidine.

[0081] Isotopic enrichment (*e.g.*, deuteration) of pharmaceuticals to improve pharmacokinetics ("PK"), pharmacodynamics ("PD"), and toxicity profiles, has been demonstrated previously with some classes of drugs. *See, e.g.*, Lijinsky *et. al.*, Food Cosmet. Toxicol., 20: 393 (1982); Lijinsky *et. al.*, J. Nat. Cancer Inst., 69: 1127 (1982); Mangold *et. al.*, Mutation Res. 308: 33 (1994); Gordon *et. al.*, Drug Metab. Dispos., 15: 589 (1987); Zello

et. al., Metabolism, 43: 487 (1994); Gately *et. al.*, J. Nucl. Med., 27: 388 (1986); Wade, D., Chem. Biol. Interact. 117: 191 (1999).

[0082] Without being limited by any particular theory, isotopic enrichment of a drug can be used, for example, to: (1) reduce or eliminate unwanted metabolites; (2) increase the half-life of the parent drug; (3) decrease the number of doses needed to achieve a desired effect; (4) decrease the amount of a dose necessary to achieve a desired effect; (5) increase the formation of active metabolites, if any are formed; and/or (6) decrease the production of deleterious metabolites in specific tissues and/or create a more effective drug and/or a safer drug for combination therapy, whether the combination therapy is intentional or not.

[0083] Replacement of an atom for one of its isotopes may often result in a change in the reaction rate of a chemical reaction. This phenomenon is known as the Kinetic Isotope Effect ("KIE"). For example, if a C–H bond is broken during a rate-determining step in a chemical reaction (*i.e.* the step with the highest transition state energy), substitution of a deuterium for that hydrogen will cause a decrease in the reaction rate and the process will slow down. This phenomenon is known as the Deuterium Kinetic Isotope Effect ("DKIE"). *See, e.g,* Foster *et al.*, Adv. Drug Res., vol. 14, pp. 1-36 (1985); Kushner *et al.*, Can. J. Physiol. Pharmacol., vol. 77, pp. 79-88 (1999).

[0084] The magnitude of the DKIE can be expressed as the ratio between the rates of a given reaction in which a C–H bond is broken, and the same reaction where deuterium is substituted for hydrogen. The DKIE can range from about 1 (no isotope effect) to very large numbers, such as 50 or more, meaning that the reaction can be fifty, or more, times slower when deuterium is substituted for hydrogen. Without being limited by a particular theory, high DKIE values may be due in part to a phenomenon known as tunneling, which is a consequence of the uncertainty principle. Tunneling is ascribed to the small mass of a hydrogen atom, and occurs because transition states involving a proton can sometimes form in the absence of the required activation energy. Because deuterium has more mass than hydrogen, it statistically has a much lower probability of undergoing this phenomenon.

[0085] Tritium ("T") is a radioactive isotope of hydrogen, used in research, fusion reactors, neutron generators and radiopharmaceuticals. Tritium is a hydrogen atom that has 2 neutrons in the nucleus and has an atomic weight close to 3. It occurs naturally in the environment in very low concentrations, most commonly found as T_2O. Tritium decays slowly (half-life = 12.3 years) and emits a low energy beta particle that cannot penetrate the outer layer of human skin. Internal exposure is the main hazard associated with this isotope,

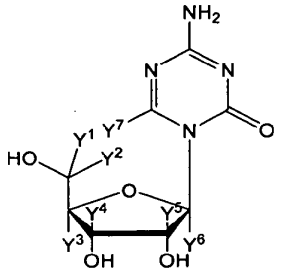
yet it must be ingested in large amounts to pose a significant health risk. As compared with deuterium, a lesser amount of tritium must be consumed before it reaches a hazardous level. Substitution of tritium ("T") for hydrogen results in yet a stronger bond than deuterium and gives numerically larger isotope effects.

[0086] Similarly, substitution of isotopes for other elements, including, but not limited to, ^{13}C or ^{14}C for carbon, ^{33}S, ^{34}S, or ^{36}S for sulfur, ^{15}N for nitrogen, and ^{17}O or ^{18}O for oxygen, may lead to an analogous kinetic isotope effect.

[0087] The animal body expresses a variety of enzymes for the purpose of eliminating foreign substances, such as therapeutic agents, from its circulation system. Examples of such enzymes include the cytochrome P450 enzymes ("CYPs"), esterases, proteases, reductases, dehydrogenases, and monoamine oxidases, to react with and convert these foreign substances to more polar intermediates or metabolites for renal excretion. Some of the most common metabolic reactions of pharmaceutical compounds involve the oxidation of a carbon-hydrogen (C–H) bond to either a carbon-oxygen (C–O) or carbon-carbon (C–C) pi-bond. The resultant metabolites may be stable or unstable under physiological conditions, and can have substantially different pharmacokinetic, pharmacodynamic, and acute and long-term toxicity profiles relative to the parent compounds. For many drugs, such oxidations are rapid. As a result, these drugs often require the administration of multiple or high daily doses.

[0088] Isotopic enrichment at certain positions of a compound provided herein may produce a detectable KIE that affects the pharmacokinetic, pharmacologic, and/or toxicological profiles of a compound provided herein in comparison with a similar compound having a natural isotopic composition. In one embodiment, the deuterium enrichment is performed on the site of C–H bond cleavage during metabolism.

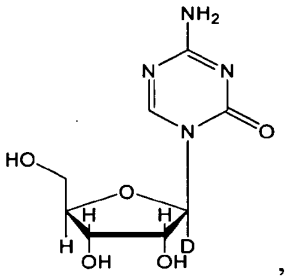
[0089] Certain embodiments herein provide deuterium enriched 5-azacytidine analogs, wherein one or more hydrogen(s) in the 5-azacytidine molecule is/are isotopically enriched with deuterium. In certain embodiments, provided herein are compounds of formula (I):



(I),

wherein one or more Y atom(s) (*i.e.*, Y^1, Y^2, Y^3, Y^4, Y^5, Y^6, and Y^7) is/are hydrogen(s) isotopically enriched with deuterium, and any remaining Y atom(s) is/are non-enriched hydrogen atom(s). In particular embodiments, one, two, three, four, five, six, or seven of the indicated Y atom(s) is/are isotopically enriched with deuterium, and any remaining Y atom(s) is/are non-enriched hydrogen(s).

[0090] In certain embodiments, one or more Y atoms on the ribose moiety of Compound (I) are deuterium-enriched. Particular examples include, but are not limited to, the following compounds, in which the label "D" indicates a deuterium-enriched atomic position, *i.e.*, a sample comprising the given compound has a deuterium enrichment at the indicated position(s) above the natural abundance of deuterium:



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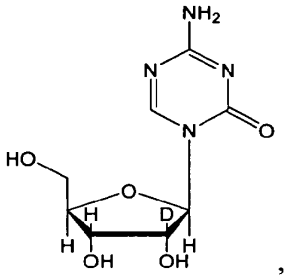
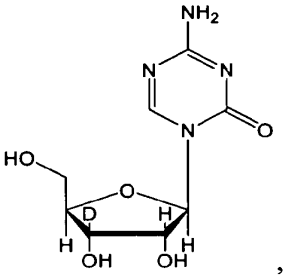


I-2



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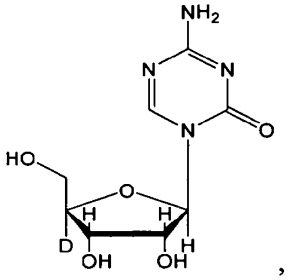

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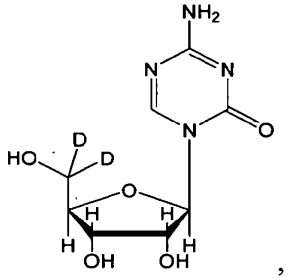

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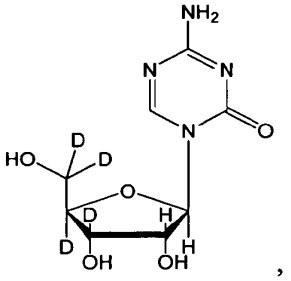
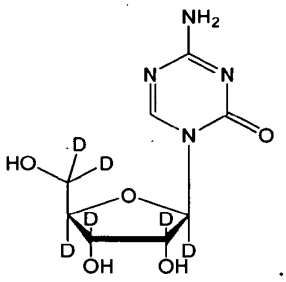


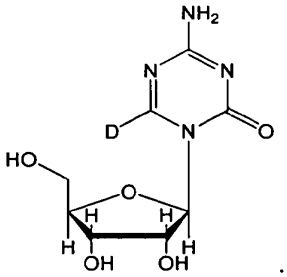
I-6

and



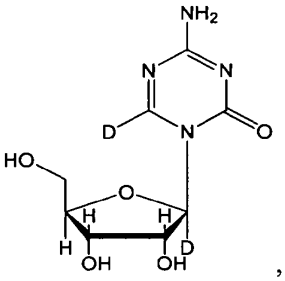
I-7

[0091] In certain embodiments, the Y atom on the 5-azacytosine moiety of Compound (I) is deuterium-enriched. Particular example includes the following compound, in which the label "D" indicates a deuterium-enriched atomic position, *i.e.*, a sample comprising the given compound has a deuterium enrichment at the indicated position(s) above the natural abundance of deuterium:



I-8

[0092] In certain embodiments, one or more Y atoms on the ribose moiety and the Y atom on the 5-azacytosine moiety of Compound (I) are deuterium-enriched. Particular examples include, but are not limited to, the following compounds, in which the label "D" indicates a deuterium-enriched atomic position, *i.e.*, a sample comprising the given compound has a deuterium enrichment at the indicated position(s) above the natural abundance of deuterium:



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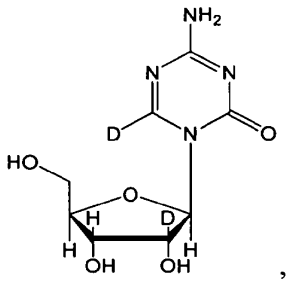
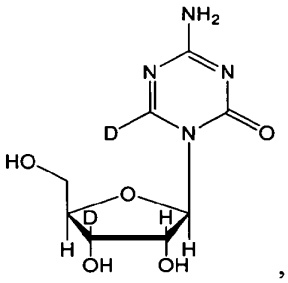
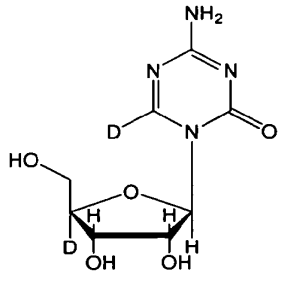


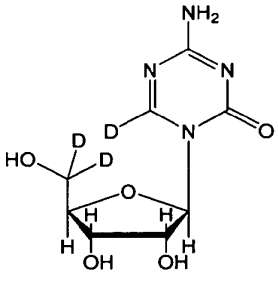
I-10



I-11



I-12



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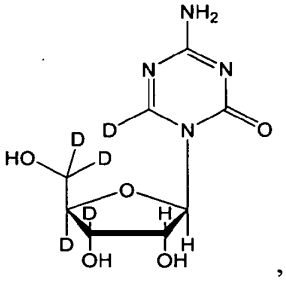
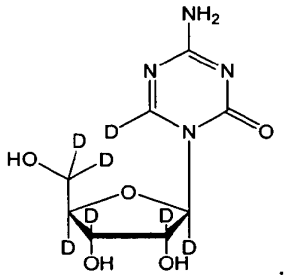


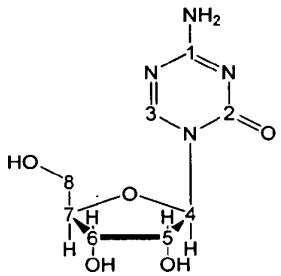
I-14

and  .

I-15

[0093] It is understood that one or more deuterium(s) may exchange with hydrogen under physiological conditions.

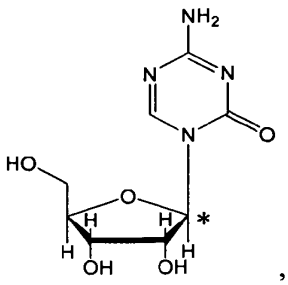
[0094] Certain embodiments herein provide carbon-13 enriched analogs of 5-azacytidine, wherein one or more carbon(s) in the 5-azacytidine molecule is/are isotopically enriched with carbon-13. In certain embodiments, provided herein are compounds of formula (II):



(II),

wherein one or more of 1, 2, 3, 4, 5, 6, 7, or 8 is/are carbon atom(s) isotopically enriched with carbon-13, and any remaining atom(s) of 1, 2, 3, 4, 5, 6, 7, or 8 is/are non-enriched carbon atom(s). In particular embodiments, one, two, three, four, five, six, seven, or eight carbon atom(s) (*i.e.*, atoms 1, 2, 3, 4, 5, 6, 7, and 8) is/are isotopically enriched with carbon-13, and any remaining carbon atom(s) is/are non-enriched.

[0095] In certain embodiments, one or more carbon atom(s) of the ribose moiety of Compound (II) are enriched with carbon-13. Particular examples include, but are not limited to, the following compounds, in which the asterisk ("*") indicates a carbon-13 enriched atomic position, *i.e.*, a sample comprising the given compound has a carbon-13 enrichment at the indicated position(s) above the natural abundance of carbon-13: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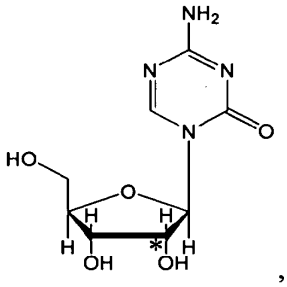
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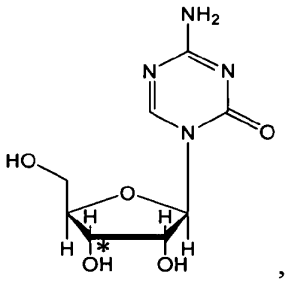
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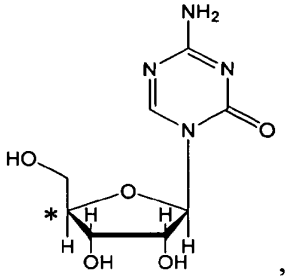
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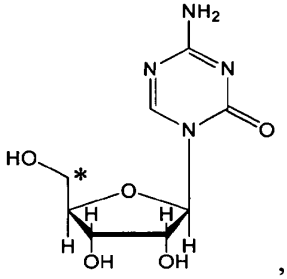
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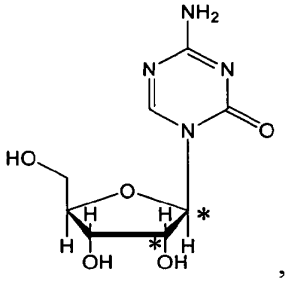
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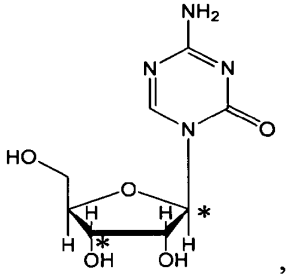
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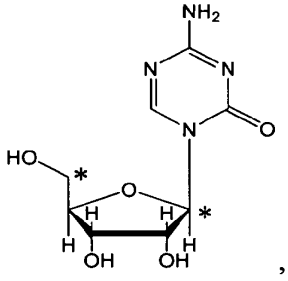
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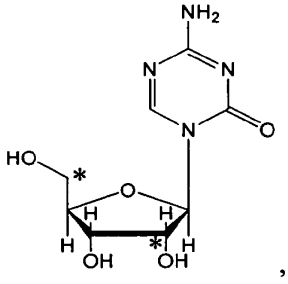
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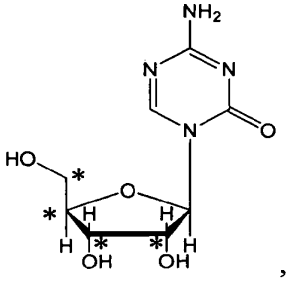
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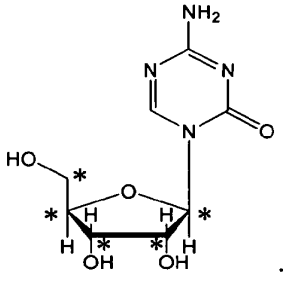
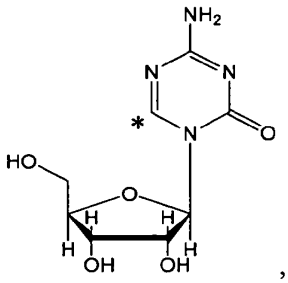
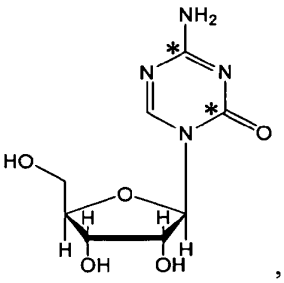
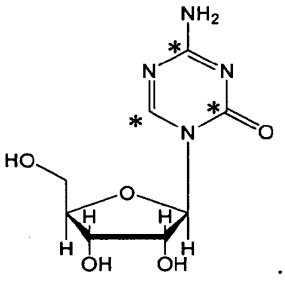

. II-11

[0096] In certain embodiments, one or more carbon atom(s) of the 5-azacytosine moiety of Compound (II) are enriched with carbon-13. Particular examples include, but are not limited to, the following compounds, in which the asterisk "*" indicates a carbon-13 enriched

atomic position, *i.e.*, a sample comprising the given compound has a carbon-13 enrichment at the indicated position(s) above the natural abundance of carbon-13:



, 

, and 

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II-12 **II-13** **II-14**

[0097] In certain embodiments, one or more carbon atoms on the ribose moiety and one or more carbon atoms on the 5-azacytosine moiety of Compound (II) are enriched with carbon-13, *i.e.*, any combination of carbon-13 enrichment for the ribose moiety and carbon-13 enrichment for the azacitosine moiety is encompassed herein.

[0098] In certain embodiments, one or more hydrogen(s) is/are enriched with deuterium(s) and one or more carbon(s) is/are enriched with carbon-13, *i.e.*, any combination of deuterium enrichment and carbon-13 enrichment of 5-azacytidine is encompassed herein.

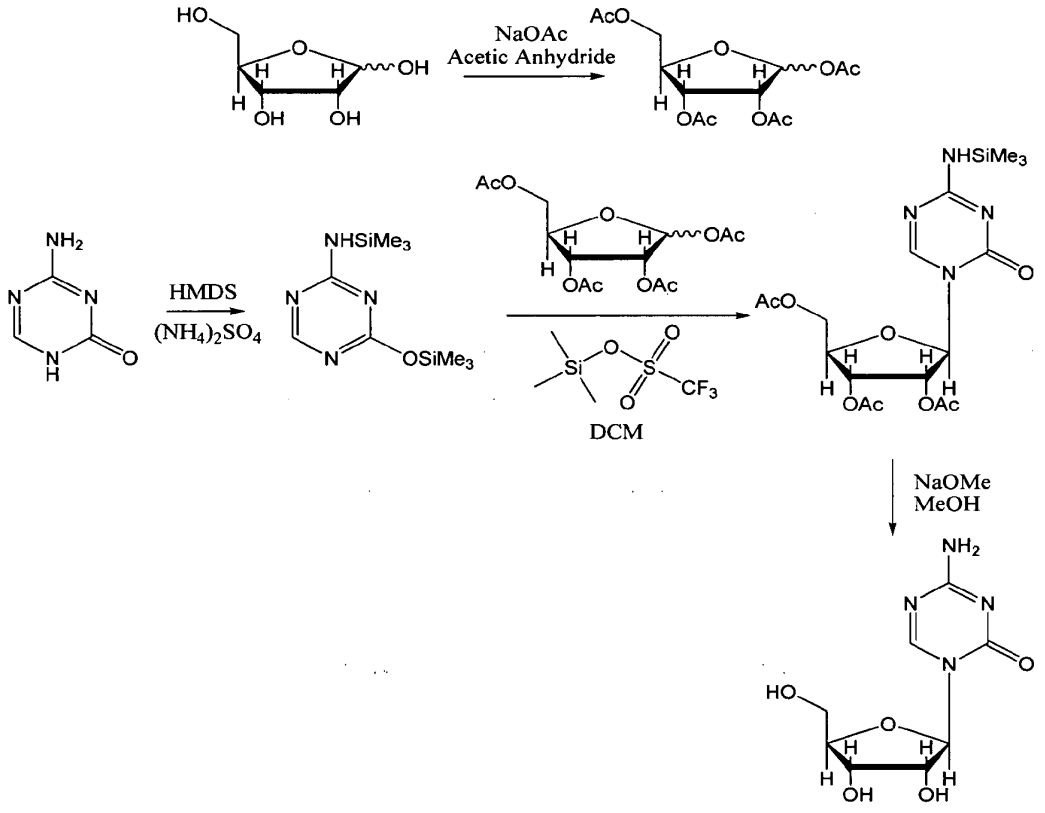
3. Synthesis of Isotopically Enriched Cytidine Analogs

[0099] The compounds described herein may be synthesized using any method known to one of ordinary skill in the art. For example, particular compounds described herein are synthesized using standard synthetic organic chemistry techniques known to those of ordinary skill in the art. In some embodiments, known procedures for the synthesis of 5-azacytidine are employed, wherein one or more of the reagents, starting materials, precursors, or intermediates are replaced by one or more isotopically-enriched reagents, starting materials, precursors, or intermediates, including but not limited to one or more deuterium-enriched reagents, starting materials, precursors, or intermediates, and/or one or more carbon-13-enriched reagents, starting materials, precursors, or intermediates. Isotopically enriched reagents, starting materials, precursors, or intermediates are commercially available or may be prepared by routine chemical reactions known to one of skill in the art. In some embodiments, the routes are based on those disclosed in U.S. Patent No. 7,038,038, which is incorporated herein by reference in its entirety.

[00100] In certain embodiments, a suitable isotopically enriched starting material, such as a deuterium-enriched ribose, a deuterium-enriched 5-azacytosine, a carbon-13-enriched

ribose, and/or a carbon-13-enriched 5-azacytosine, may be employed as the starting material in the following general scheme to prepare the corresponding deuterium and/or carbon-13 enriched 5-azacytidine (See Scheme 1). Following the procedures in U.S. Patent No. 7,038,038, 5-azacytosine is treated with hexamethyldisilazane (HMDS) to render a silylated 5-azacytosine. Tetraacetyl-D-ribose is prepared by reacting D-ribose with sodium acetate in acetic anhydride, following the procedures in Brown *et al.*, Biochemical Preparations, **1955**, *4*, 70-76. The silylated 5-azacytosine is coupled to tetraacetyl-D-ribose in the presence of TMS-triflate, and the resulting protected 5-azacytidine is treated with sodium methoxide in methanol to yield 5-azacytidine. *See* U.S. Patent No. 7,038,038.

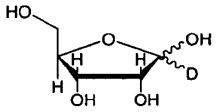
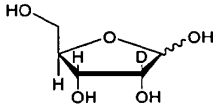
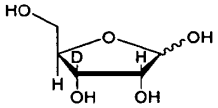
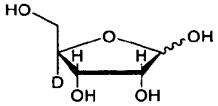
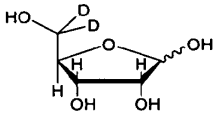
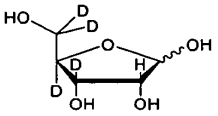
Scheme 1



[00101] In some embodiments, one or more hydrogen positions in the ribose portion of 5-azacytidine are enriched with deuterium. Such 5-azacytidine analogs may be prepared following Scheme 1 from a suitable deuterium-enriched ribose, purchased from a commercial

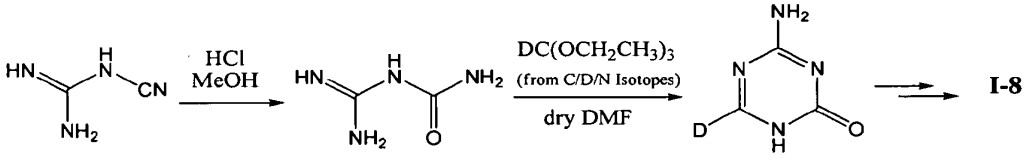
source or prepared following literature procedures. Specific examples of deuterium-enriched ribose starting material include, but are not limited to, the following compounds listed in Table 1, which may be converted to the corresponding deuterium-enriched 5-azacytidine analogs.

TABLE 1

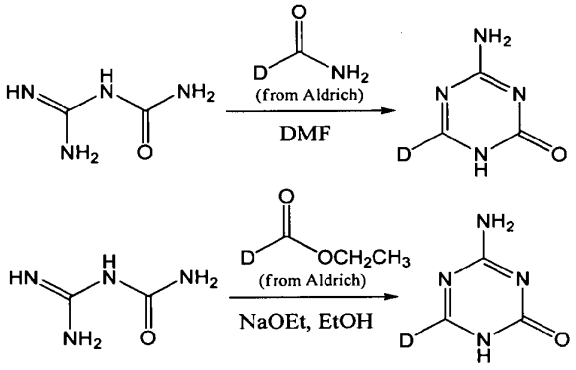
Starting Material	Structure	Source/Reference	5-Azacytidine Product
D-Ribose-1-D		Cambridge Isotope Lab.	I-1
D-Ribose-2-D		Cambridge Isotope Lab.	I-2
D-Ribose-3-D		Omicron Biochemicals, Inc.	I-3
D-Ribose-4-D		Omicron Biochemicals, Inc.	I-4
D-Ribose-5,5'-D_2		Omicron Biochemicals, Inc.	I-5
D-Ribose-3,4,5,5'-D_4		Prepared following the procedures in J. Am. Chem. Soc. **1996**, *118*, 7929-7940.	I-6

[00102] In other embodiments, the hydrogen position on the 5-azacytosine ring of 5-azacytidine is enriched with deuterium. Such 5-azacytidine analog may be prepared, *e.g.*, from deuterated 5-azacytosine following Scheme 1. The deuterated 5-azacytosine may be prepared, *e.g.*, from suitable deuterated reagents as shown in Scheme 2. *See e.g.*, Grundmann *et al.*, Chem. Ber. **1954**, *87*, 19-24; Piskala *et al.*, in Zorbach and Tipson (eds.) Synthetic Procedures in Nucleic Acid Chemistry, Vol. 1, Wiley Interscience, New York, **1968**, 107-108; Piskala, Collect. Czech. Chem. Comm. 1967, 32, 3966-3976.

Scheme 2



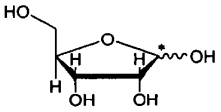
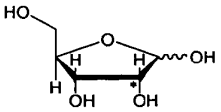
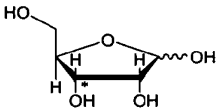
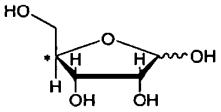
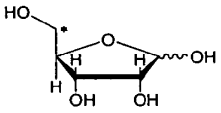
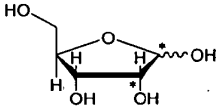
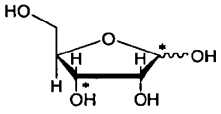
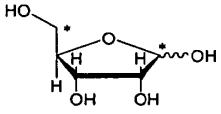
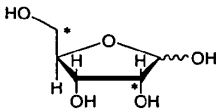
Alternative conditions for preparing 5-azacytosine:

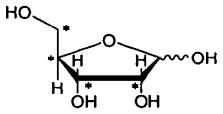
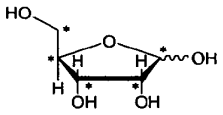


[00103] In other embodiments, both the hydrogen position on the 5-azacytosine ring and one or more hydrogen positions in the ribose portion of 5-azacytidine are enriched with deuterium. Such 5-azacytidine analogs may be prepared, *e.g.*, following Scheme 1, coupling a suitable deuterated ribose starting materials with deuterated 5-azacytosine. For example, compounds I-9, I-10, I-11, I-12, I-13, and I-14 may be prepared from the corresponding deuterated ribose starting material listed in Table 1, and deuterated 5-azacytosine prepared according to Scheme 2.

[00104] In some embodiments, one or more carbon atoms in the ribose portion of 5-azacytidine are enriched with carbon-13. Such 5-azacytidine analogs may be prepared following Scheme 1 from a suitable carbon-13-enriched ribose, purchased from a commercial source or prepared following literature procedures. Specific examples of carbon-13-enriched ribose starting material include, but are not limited to, the following compounds listed in Table 2, which may be converted to the corresponding carbon-13-enriched 5-azacytidine analogs. (The asterisk "*" indicates a carbon-13 enriched atomic position)

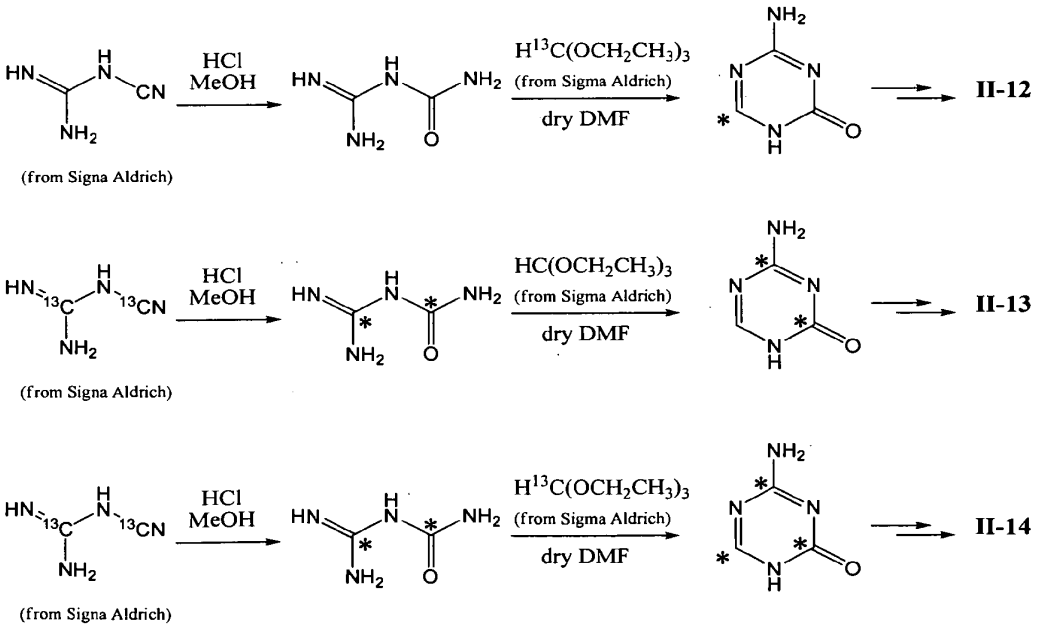
TABLE 2

Starting Material	Structure	Source/Reference	5-Azacytidine Product
D-Ribose-1-^{13}C		Sigma Aldrich	II-1
D-Ribose-2-^{13}C		Sigma Aldrich	II-2
D-Ribose-3-^{13}C		Omicron Biochemicals, Inc.	II-3
D-Ribose-4-^{13}C		Omicron Biochemicals, Inc.	II-4
D-Ribose-5-^{13}C		Cambridge Isotope Lab.	II-5
D-Ribose-1,2-$^{13}C_2$		Sigma Aldrich	II-6
D-Ribose-1,3-$^{13}C_2$		Omicron Biochemicals, Inc.	II-7
D-Ribose-1,5-$^{13}C_2$		Omicron Biochemicals, Inc.	II-8
D-Ribose-2,5-$^{13}C_2$		Omicron Biochemicals, Inc.	II-9

D-Ribose-2,3,4,5-$^{13}C_4$		Sigma Aldrich	II-10
D-Ribose-1,2,3,4,5-$^{13}C_5$		Cambridge Isotope Lab.	II-11

[00105] In other embodiments, one or more carbon atoms in the 5-azacytosine ring are enriched with carbon-13. Such 5-azacytidine analogs may be prepared from a carbon-13-enriched 5-azacytosine following Scheme 1. The carbon-13 enriched 5-azacytosine intermediates may be prepared from suitable carbon-13 enriched reagents as shown in Scheme 3. *See e.g.*, Grundmann *et al.*, Chem. Ber. **1954**, *87*, 19-24; Piskala *et al.*, in Zorbach and Tipson (eds.) Synthetic Procedures in Nucleic Acid Chemistry, Vol. 1, Wiley Interscience, New York, **1968**, 107-108; Piskala, Collect. Czech. Chem. Comm. 1967, 32, 3966-3976.

Scheme 3



[00106] In other embodiments, one or more carbon positions on the 5-azacytosine ring and one or more carbon positions in the ribose portion of 5-azacytidine are enriched with carbon-

13. Such 5-azacytidine analogs may be prepared following Scheme 1, coupling a suitable carbon-13-enriched ribose starting materials with a suitable carbon-13-enriched 5-azacytosine. For example, compounds may be prepared from a carbon-13-enriched ribose starting material listed in Table 2, and carbon-13-enriched 5-azacytosine prepared according to Scheme 3.

[00107] The routes and methods described above may be modified to provide an isotopolougue of 5-azacytidine having both deuterium enrichment and carbon-13 enrichment.

C. Pharmaceutical Formulations

1. Overview

[00108] Embodiments herein encompass pharmaceutical formulations and compositions comprising one or more cytidine analogs, *e.g.*, 5-azacytidine, and optionally a permeation enhancer, wherein the formulations and compositions are prepared for oral administration. In a particular embodiment, the formulations and compositions are prepared for release of the cytidine analog substantially in the stomach. In specific embodiments, the cytidine analogs, *e.g.*, 5-azacytidine, and the pharmaceutical formulations and compositions are used for treating diseases and disorders associated with abnormal cell proliferation, wherein the cytidine analogs, the formulations and compositions are prepared for oral administration, preferably for release of the cytidine analogs substantially in the stomach. Particular embodiments relate to the use of one or more cytidine analogs, *e.g.*, 5-azacytidine, for the preparation of pharmaceutical formulations and compositions for treating particular medical indications, as provided herein. The pharmaceutical formulations and compositions comprising cytidine analogs provided herein are intended for oral delivery of the cytidine analog in subjects in need thereof. Oral delivery formats include, but are not limited to, tablets, capsules, caplets, solutions, suspensions, and syrups, and may also comprise a plurality of granules, beads, powders or pellets that may or may not be encapsulated. Such formats may also be referred to herein as the "drug core" which contains the cytidine analog.

[00109] Particular embodiments herein provide solid oral dosage forms that are tablets or capsules. In certain embodiments, the formulation is a tablet comprising a cytidine analog. In certain embodiments, the formulation is a capsule comprising a cytidine analog. In certain embodiments, the tablets or capsules provided herein optionally comprise one or more excipients, such as, for example, glidants, diluents, lubricants, colorants, disintegrants, granulating agents, binding agents, polymers, and coating agents. In certain embodiments, the formulation is an immediate release tablet. In certain embodiments, the formulation is a

controlled release tablet releasing the API, *e.g.*, substantially in the stomach. In certain embodiments, the formulation is a hard gelatin capsule. In certain embodiments, the formulation is a soft gelatin capsule. In certain embodiments, the capsule is a hydroxypropyl methylcellulose (HPMC) capsule. In certain embodiments, the formulation is an immediate release capsule. In certain embodiments, the formulation is an immediate or controlled release capsule releasing the API, *e.g.*, substantially in the stomach. In certain embodiments, the formulation is a rapidly disintegrating tablet that dissolves substantially in the mouth following administration. In certain embodiments, embodiments herein encompass the use of cytidine analogs, e.g., 5-azacytidine, for the preparation of a pharmaceutical composition for treating a disease associated with abnormal cell proliferation, wherein the composition is prepared for oral administration.

2. Performance of Certain Dosage Forms Provided Herein

[00110] In certain embodiments, the formulations comprising the cytidine analogs, such as, for example, 5-azacytidine, effect an immediate release of the API upon oral administration. In particular embodiments, the formulations comprising the cytidine analogs, such as, for example, 5-azacytidine, comprise a therapeutically or prophylactically effective amount of the cytidine analog (and, optionally, one or more excipients) and effect an immediate release of the API upon oral administration.

[00111] In certain embodiments, the formulations comprising the cytidine analogs, such as, for example, 5-azacytidine, effect a controlled release of the API substantially in the stomach upon oral administration. In certain embodiments, the formulations comprising the cytidine analogs, such as, for example, 5-azacytidine, comprise a therapeutically or prophylactically effective amount of the cytidine analog and a drug release controlling component which is capable of releasing the cytidine analog substantially in the stomach. In certain embodiments, matrices (*e.g.*, polymer matrices) may be employed in the formulation to control the release of the cytidine analog. In certain embodiments, coatings and/or shells may be employed in the formulation to control the release of the cytidine analog in the substantially in the stomach.

[00112] In certain embodiments, the formulations comprising the cytidine analogs, such as, for example, 5-azacytidine, release the API substantially in the stomach upon oral administration. In certain embodiments, the formulations effect an immediate release of the cytidine analog upon oral administration. In certain embodiments, the formulations optionally further comprises a drug release controlling component, wherein the drug release

controlling component is adjusted such that the release of the cytidine analog occurs substantially in the stomach. In particular embodiments, the drug release controlling component is adjusted such that the release of the cytidine analog is immediate and occurs substantially in the stomach. In particular embodiments, the drug release controlling component is adjusted such that the release of the cytidine analog is sustained and occurs substantially in the stomach. In certain embodiments, the formulation of the cytidine analog, such as, for example, 5-azacytidine, releases the API substantially in the stomach, and, subsequently, releases the remainder of the API in the intestine upon oral administration.

[00113] Methods by which skilled practitioners can assess where a drug is released in the gastrointestinal tract of a subject are known in the art, and include, for example, scintigraphic studies, testing in a bio-relevant medium which simulates the fluid in relevant portions of the gastrointestinal tract, among other methods.

[00114] Particular embodiments herein provide pharmaceutical formulations (*e.g.*, immediate release oral formulations and/or formulations that release the API substantially in the stomach) comprising a cytidine analog (*e.g.*, 5-azacytidine) that achieve a particular exposure in the subject to which the formulation is orally administered, as compared to a SC dose of the same cytidine analog. Particular embodiments provide oral formulations that achieve an exposure of at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or about 100%, as compared to a SC dose.

[00115] In certain embodiments, the formulation (*e.g.*, immediate release oral formulation and/or formulation that release the API substantially in the stomach) comprising the cytidine analog, such as, for example, 5-azacytidine, renders a certain percentage of the cytidine analog in the formulation systemically bioavailable upon oral administration. In certain embodiments, after the subject is orally administered the formulation, the cytidine analog in the formulation is absorbed substantially in the stomach, and becomes available to the body through systemic exposure. In particular embodiments, the oral bioavailability of a formulation comprising a cytidine analog provided herein is, *e.g.*, greater than about 1%, greater than about 5%, greater than about 10%, greater than about 15%, greater than about 20%, greater than about 25%, greater than about 30%, greater than about 35%, greater than about 40%, greater than about 45%, greater than about 50%, greater than about 55%, greater

than about 60%, greater than about 65%, greater than about 70%, greater than about 75%, greater than about 80%, greater than about 85%, greater than about 90%, greater than about 95%, or about 100%, of the total amount of the cytidine analog in the formulation.

[00116] Methods by which skilled practitioners can assess the oral bioavailability of a drug formulation in a subject are known in the art. Such methods, include, for example, comparing certain dosing-related parameters, such as, but not limited to, maximum plasma concentration ("Cmax"), time to maximum plasma concentration ("Tmax"), or area-under-the-curve ("AUC") determinations.

[00117] Particular embodiments herein provide pharmaceutical formulations (*e.g.*, immediate release oral formulations and/or formulations that release the API substantially in the stomach) comprising a cytidine analog (*e.g.*, 5-azacytidine) that achieve a particular AUC value (*e.g.*, AUC(0-t) or AUC(0-∞)) in the subject (*e.g.*, human) to which the formulation is orally administered. Particular embodiments provide oral formulations that achieve an AUC value of at least about 25 ng-hr/mL, at least about 50 ng-hr/mL, at least about 75 ng-hr/mL, at least about 100 ng-hr/mL, at least about 150 ng-hr/mL, at least about 200 ng-hr/mL, at least about 250 ng-hr/mL, at least about 300 ng-hr/mL, at least about 350 ng-hr/mL, at least about 400 ng-hr/mL, at least about 450 ng-hr/mL, at least about 500 ng-hr/mL, at least about 550 ng-hr/mL, at least about 600 ng-hr/mL, at least about 650 ng-hr/mL, at least about 700 ng-hr/mL, at least about 750 ng-hr/mL, at least about 800 ng-hr/mL, at least about 850 ng-hr/mL, at least about 900 ng-hr/mL, at least about 950 ng-hr/mL, at least about 1000 ng-hr/mL, at least about 1100 ng-hr/mL, at least about 1200 ng-hr/mL, at least about 1300 ng-hr/mL, at least about 1400 ng-hr/mL, at least about 1500 ng-hr/mL, at least about 1600 ng-hr/mL, at least about 1700 ng-hr/mL, at least about 1800 ng-hr/mL, at least about 1900 ng-hr/mL, at least about 2000 ng-hr/mL, at least about 2250 ng-hr/mL, or at least about 2500 ng-hr/mL. In particular embodiments, the AUC determination is obtained from a time-concentration pharmacokinetic profile obtained from the blood samples of animals or human volunteers following dosing.

[00118] Particular embodiments herein provide pharmaceutical formulations (*e.g.*, immediate release oral formulations and/or formulations that release the API substantially in the stomach) comprising a cytidine analog (*e.g.*, 5-azacytidine) that achieve a particular maximum plasma concentration ("Cmax") in the subject to which the formulation is orally administered. Particular embodiments provide oral formulations that achieve a Cmax of the cytidine analog of at least about 25 ng/mL, at least about 50 ng/mL, at least about 75 ng/mL,

at least about 100 ng/mL, at least about 150 ng/mL, at least about 200 ng/mL, at least about 250 ng/mL, at least about 300 ng/mL, at least about 350 ng/mL, at least about 400 ng/mL, at least about 450 ng/mL, at least about 500 ng/mL, at least about 550 ng/mL, at least about 600 ng/mL, at least about 650 ng/mL, at least about 700 ng/mL, at least about 750 ng/mL, at least about 800 ng/mL, at least about 850 ng/mL, at least about 900 ng/mL, at least about 950 ng/mL, at least about 1000 ng/mL, at least about 1100 ng/mL, at least about 1200 ng/mL, at least about 1300 ng/mL, at least about 1400 ng/mL, at least about 1500 ng/mL, at least about 1600 ng/mL, at least about 1700 ng/mL, at least about 1800 ng/mL, at least about 1900 ng/mL, at least about 2000 ng/mL, at least about 2250 ng/mL, or at least about 2500 ng/mL.

[00119] Particular embodiments herein provide pharmaceutical formulations (*e.g.*, immediate release oral formulations and/or formulations that release the API substantially in the stomach) comprising a cytidine analog (*e.g.*, 5-azacytidine) that achieve a particular time to maximum plasma concentration ("Tmax") in the subject to which the formulation is orally administered. Particular embodiments provide oral formulations that achieve a Tmax of the cytidine analog of less than about 10 min., less than about 15 min., less than about 20 min., less than about 25 min., less than about 30 min., less than about 35 min., less than about 40 min., less than about 45 min., less than about 50 min., less than about 55 min., less than about 60 min., less than about 65 min., less than about 70 min., less than about 75 min., less than about 80 min., less than about 85 min., less than about 90 min., less than about 95 min., less than about 100 min., less than about 105 min., less than about 110 min., less than about 115 min., less than about 120 min., less than about 130 min., less than about 140 min., less than about 150 min., less than about 160 min., less than about 170 min., less than about 180 min., less than about 190 min., less than about 200 min., less than about 210 min., less than about 220 min., less than about 230 min., or less than about 240 min. In particular embodiments, the Tmax value is measured from the time at which the formulation is orally administered.

[00120] Particular embodiments herein provide oral dosage forms comprising a cytidine analog, wherein the oral dosage forms have an enteric coating. Particular embodiments provide a permeable or partly permeable (*e.g.*, "leaky") enteric coating with pores. In particular embodiments, the permeable or partly permeable enteric-coated tablet releases the 5-azacytidine in an immediate release manner substantially in the stomach.

3. Design of Certain Dosage Forms Provided Herein

[00121] Provided herein are dosage forms designed to maximize the absorption and/or efficacious delivery of certain cytidine analogs, *e.g.*, 5-azacytidine, upon oral administration,

e.g., for release substantially in the stomach. Accordingly, certain embodiments herein provide a solid oral dosage form of a cytidine analog, such as, for example, 5-azacytidine, using pharmaceutical excipients designed for immediate release of the API upon oral administration, *e.g.*, substantially in the stomach. Particular immediate release formulations comprise a specific amount of a cytidine analog and optionally one or more excipients. In certain embodiments, the formulation may be an immediate release tablet or an immediate release capsule (such as, *e.g.*, an HPMC capsule).

[00122] Provided herein are methods of making the formulations provided herein comprising the cytidine analogs provided herein (*e.g.*, immediate release oral formulations and/or formulations that release the API substantially in the stomach). In particular embodiments, the formulations provided herein may be prepared using conventional methods known to those skilled in the field of pharmaceutical formulation, as described, *e.g.*, in pertinent textbooks. *See, e.g.*, REMINGTON, THE SCIENCE AND PRACTICE OF PHARMACY, 20th Edition, Lippincott Williams & Wilkins, (2000); ANSEL *et al.*, PHARMACEUTICAL DOSAGE FORMS AND DRUG DELIVERY SYSTEMS, 7th Edition, Lippincott Williams & Wilkins, (1999); GIBSON, PHARMACEUTICAL PREFORMULATION AND FORMULATION, CRC Press (2001).

[00123] In particular embodiments, formulations provided herein (*e.g.*, immediate release oral formulations, formulations that release the API substantially in the stomach, or rapidly disintegrating formulations that dissolve substantially in the mouth) comprise a cytidine analog, such as, for example, 5-azacytidine, in a specific amount. In particular embodiments, the specific amount of the cytidine analog in the formulation is, *e.g.*, about 10 mg, about 20 mg, about 40 mg, about 60 mg, about 80 mg, about 100 mg, about 120 mg, about 140 mg, about 160 mg, about 180 mg, about 200 mg, about 220 mg, least about 240 mg, about 260 mg, about 280 mg, about 300 mg, about 320 mg, about 340 mg, about 360 mg, about 380 mg, about 400 mg, about 420 mg, about 440 mg, about 460 mg, about 480 mg, about 500 mg, about 600 mg, about 700 mg, about 800 mg, about 900 mg, about 1000 mg, about 1100 mg, about 1200 mg, about 1300 mg, about 1400 mg, about 1500 mg, about 1600 mg, about 1700 mg, about 1800 mg, about 1900 mg, about 2000 mg, about 2100 mg, about 2200 mg, about 2300 mg, about 2400 mg, about 2500 mg, about 3000 mg, about 4000 mg, or about 5000 mg. In particular embodiments, the specific amount of the cytidine analog in the formulation is, *e.g.*, at least about 10 mg, at least about 20 mg, at least about 40 mg, at least about 60 mg, at least about 80 mg, at least about 100 mg, at least about 120 mg, at least about 140 mg, at least about 160 mg, at least about 180 mg, at least about 200 mg, at least about 220 mg, at least

about 240 mg, at least about 260 mg, at least about 280 mg, at least about 300 mg, at least about 320 mg, at least about 340 mg, at least about 360 mg, at least about 380 mg, at least about 400 mg, at least about 420 mg, at least about 440 mg, at least about 460 mg, at least about 480 mg, at least about 500 mg, at least about 600 mg, at least about 700 mg, at least about 800 mg, at least about 900 mg, at least about 1000 mg, at least about 1100 mg, at least about 1200 mg, at least about 1300 mg, at least about 1400 mg, at least about 1500 mg, at least about 1600 mg, at least about 1700 mg, at least about 1800 mg, at least about 1900 mg, at least about 2000 mg, at least about 2100 mg, at least about 2200 mg, at least about 2300 mg, at least about 2400 mg, at least about 2500 mg, at least about 3000 mg, at least about 4000 mg, or at least about 5000 mg.

[00124] In certain embodiments, the formulation is a tablet, wherein the tablet is manufactured using standard, art-recognized tablet processing procedures and equipment. In certain embodiments, the method for forming the tablets is direct compression of a powdered, crystalline and/or granular composition comprising the cytidine analog, alone or in combination with one or more excipients, such as, for example, carriers, additives, polymers, or the like. In certain embodiments, as an alternative to direct compression, the tablets may be prepared using wet granulation or dry granulation processes. In certain embodiments, the tablets are molded rather than compressed, starting with a moist or otherwise tractable material. In certain embodiments, compression and granulation techniques are used.

[00125] In certain embodiments, the formulation is a capsule, wherein the capsules may be manufactured using standard, art-recognized capsule processing procedures and equipments. In certain embodiments, soft gelatin capsules may be prepared in which the capsules contain a mixture of the cytidine analog and vegetable oil or non-aqueous, water miscible materials such as, for example, polyethylene glycol and the like. In certain embodiments, hard gelatin capsules may be prepared containing granules of the cytidine analog in combination with a solid pulverulent carrier, such as, for example, lactose, saccharose, sorbitol, mannitol, potato starch, corn starch, amylopectin, cellulose derivatives, or gelatin. In certain embodiments, a hard gelatin capsule shell may be prepared from a capsule composition comprising gelatin and a small amount of plasticizer such as glycerol. In certain embodiments, as an alternative to gelatin, the capsule shell may be made of a carbohydrate material. In certain embodiments, the capsule composition may additionally include polymers, colorings, flavorings and opacifiers as required. In certain embodiments, the capsule comprises HPMC.

[00126] In certain embodiments, the formulation of the cytidine analog, such as, for example, 5-azacytidine, is prepared using aqueous solvents without causing significant hydrolytic degradation of the cytidine analog. In particular embodiments, the formulation of the cytidine analog, such as, for example, 5-azacytidine, is a tablet which contains a coating applied to the drug core using aqueous solvents without causing significant hydrolytic degradation of the cytidine analog in the formulation. In certain embodiments, water is employed as the solvent for coating the drug core. In certain embodiments, the oral dosage form of the cytidine analog is a tablet containing a film coat applied to the drug core using aqueous solvents. In particular embodiments, water is employed as the solvent for film-coating. In particular embodiments, the tablet containing the cytidine analog is film-coated using aqueous solvents without effecting degradation of the pharmaceutical composition. In particular embodiments, water is used as the film coating solvent without effecting degradation of the pharmaceutical composition. In certain embodiments, an oral dosage form comprising 5-azacytidine and an aqueous film coating effects immediate drug release upon oral delivery. In certain embodiments, the oral dosage form comprising 5-azacytidine and an aqueous film coating effects controlled drug release to the upper gastrointestinal tract, *e.g.*, the stomach, upon oral administration. In particular embodiments, a tablet with an aqueous-based film coating comprises 5-azacytidine as the API.

[00127] In certain embodiments, provided herein is a controlled release pharmaceutical formulation for oral administration of a cytidine analog that releases the cytidine analog substantially in the stomach, comprising: a) a specific amount of a cytidine analog; b) a drug release controlling component for controlling the release of the cytidine analog substantially in the upper gastrointestinal tract, *e.g.*, the stomach; and c) optionally one or more excipients. In certain embodiments, the oral dosage form comprising the cytidine analog is prepared as a controlled release tablet or capsule which includes a drug core comprising the pharmaceutical composition and optional excipients. Optionally, a "seal coat" or "shell" is applied. In certain embodiments, a formulation provided herein comprising a cytidine analog provided herein is a controlled release tablet or capsule, which comprises a therapeutically effective amount of the cytidine analog, a drug release controlling component that controls the release of the cytidine analog substantially in the stomach upon oral administration, and optionally, one or more excipients.

[00128] Particular embodiments provide a drug release controlling component that is a polymer matrix, which swells upon exposure to gastric fluid to effect the gastric retention of

the formulation and the sustained release of the cytidine analog from the polymer matrix substantially in the stomach. In certain embodiments, such formulations may be prepared by incorporating the cytidine analog into a suitable polymeric matrix during formulation. Examples of such formulations are known in the art. *See, e.g.*, Shell *et al.*, U.S. Patent Publication No. 2002/0051820 (Application No. 09/990,061); Shell *et al.*, U.S. Patent Publication No. 2003/0039688 (Application No. 10/045,823); Gusler *et al.*, U.S. Patent Publication No. 2003/0104053 (Application No. 10/029,134), each of which is incorporated herein by reference in its entirety.

[00129] In certain embodiments, the drug release controlling component may comprise a shell surrounding the drug-containing core, wherein the shell releases the cytidine analog from the core by, *e.g.*, permitting diffusion of the cytidine analog from the core and promoting gastric retention of the formulation by swelling upon exposure to gastric fluids to a size that is retained in the stomach. In certain embodiments, such formulations may be prepared by first compressing a mixture of the cytidine analog and one or more excipients to form a drug core, and compressing another powdered mixture over the drug core to form the shell, or enclosing the drug core with a capsule shell made of suitable materials. Examples of such formulations are known in the art. *See, e.g.*, Berner *et al.*, U.S. Patent Publication No. 2003/0104062 Application No. 10/213,823), incorporated herein by reference in its entirety.

[00130] Certain embodiments herein provide oral dosage forms comprising a cytidine analog, wherein the dosage form contains pores in the conventional enteric coating. In particular embodiments, the oral dosage form of the cytidine analog is a tablet that contains a permeable or partly permeable (*e.g.*, "leaky") enteric coating with pores. In particular embodiments, the permeable or partly permeable enteric-coated tablet controls the release of the cytidine analog from the tablet primarily to the upper gastrointestinal tract, *e.g.*, the stomach. In particular embodiments, the permeable or partly permeable enteric-coated tablet comprises 5-azacytidine. In particular embodiments, the remainder of the cytidine analog is subsequently released beyond the stomach (*e.g.*, in the intestine).

[00131] In certain embodiments, the pharmaceutical formulation provided herein is a compressed tablet comprising a cytidine analog. In addition to the cytidine analog, the tablet optionally comprises one or more excipients, including (a) diluents or fillers, which may add necessary bulk to a formulation to prepare tablets of the desired size; (b) binders or adhesives, which may promote adhesion of the particles of the formulation, enabling a granulation to be prepared and maintaining the integrity of the final tablet; (c) disintegrants or disintegrating

agents, which, after administration, may promote breakup of the tablets to smaller particles for improved drug availability; (d) anti-adherents, glidants, lubricants or lubricating agents, which may enhance flow of the tableting material into the tablet dies, minimize wear of the punches and dies, prevent the sticking of fill material to the punches and dies, and produce tablets having a sheen; and (e) miscellaneous adjuncts such as colorants and flavorants. After compression, tablets provided herein may be coated with various materials as described herein.

[00132] In certain embodiments, the pharmaceutical formulation provided herein is a multiple compressed tablet of a cytidine analog. Multiple compressed tablets are prepared by subjecting the fill material to more than a single compression. The result may be a multiple-layered tablet or a tablet-within-a-tablet, the inner tablet being the core comprising a cytidine analog and optionally one or more excipients, and the outer portion being the shell, wherein the shell comprises one or more excipients, and may or may not contain the cytidine analog. Layered tablets may be prepared by the initial compaction of a portion of fill material in a die followed by additional fill material and compression to form two- or three-layered tablets, depending upon the number of separate fills. Each layer may contain a different therapeutic agent, separate from one another for reasons of chemical or physical incompatibility, or the same therapeutic agent for staged drug release, or simply for the unique appearance of the multiple-layered tablet. Each portion of fill may be colored differently to prepare a distinctive looking tablet. In the preparation of tablets having a compressed tablet as the inner core, special machines may be used to place the preformed tablet precisely within the die for the subsequent compression of surrounding fill material.

[00133] In certain embodiments, the compressed tablet of a cytidine analog may be coated with a colored or an uncolored sugar layer. The coating may be water-soluble and quickly dissolved after oral ingestion. The sugar coating may serve the purpose of protecting the enclosed drug from the environment and providing a barrier to an objectionable taste or smell. The sugar coating may also enhance the appearance of the compressed tablet and permit the imprinting of identifying manufacturer's information. In certain embodiments, sugar-coated tablets may be 50% larger and heavier than the original uncoated tablets. The sugar-coating of tablets may be divided into the following optional steps: (1) waterproofing and sealing (if needed); (2) sub-coating; (3) smoothing and final rounding; (4) finishing and coloring (if desired); (5) imprinting (if needed); and (6) polishing.

[00134] In certain embodiments, the compressed tablet of a cytidine analog may be film-coated. Film-coated tablets may be compressed tablets coated with a thin layer of a polymer capable of forming a skin-like film over the tablet. The film is usually colored and has the advantage to be more durable, less bulky, and less time-consuming to apply. By its composition, the coating may be designed to rupture and expose the core tablet at the desired location within the gastrointestinal tract. The film-coating process, which places a thin skin-tight coating of a plastic-like material over the compressed tablet, may produce coated tablets having essentially the same weight, shape, and size as the originally compressed tablet. The film-coating may be colored to make the tablets attractive and distinctive. Film-coating solutions may be non-aqueous or aqueous. In particular embodiments, the non-aqueous solutions may optionally contain one or more of the following types of materials to provide the desired coating to the tablets: (1) a film former capable of producing smooth, thin films reproducible under conventional coating conditions and applicable to a variety of tablet shapes, such as, for example, cellulose acetate phthalate; (2) an alloying substance providing water solubility or permeability to the film to ensure penetration by body fluids and therapeutic availability of the drug, such as, for example, polyethylene glycol; (3) a plasticizer to produce flexibility and elasticity of the coating and thus provide durability, such as, for example, castor oil; (4) a surfactant to enhance spreadability of the film during application, such as, for example, polyoxyethylene sorbitan derivatives; (5) opaquants and colorants to make the appearance of the coated tablets attractive and distinctive, such as, for example, titanium dioxide as an opaquant, and FD&C or D&C dyes as a colorant; (6) sweeteners, flavors, or aromas to enhance the acceptability of the tablet to the subject, such as, for example, saccharin as sweeteners, and vanillin as flavors and aromas; (7) a glossant to provide a luster to the tablets without a separate polishing operation, such as, for example, beeswax; and (8) a volatile solvent to allow the spread of the other components over the tablets while allowing rapid evaporation to permit an effective yet speedy operation, such as, for example, alcohol-acetone mixture. In certain embodiments, an aqueous film-coating formulation may contain one or more of the following: (1) film-forming polymer, such as, for example, cellulose ether polymers as hydroxypropyl methyl-cellulose, hydroxypropyl cellulose, and methyl-cellulose; (2) plasticizer, such as, for example, glycerin, propylene glycol, polyethylene glycol, diethyl phthalate, and dibutyl subacetate; (3) colorant and opacifier, such as, for example, FD&C or D&C lakes and iron oxide pigments; or (4) vehicle, such as, for example, water.

[00135] In certain embodiments, the compressed tablet of a cytidine analog may be compression-coated. The coating material, in the form of a granulation or powder, may be compressed onto a tablet core of drug with a special tablet press.

[00136] In certain embodiments, the pharmaceutical formulation is a gelatin-coated tablet of a cytidine analog. A gelatin-coated tablet is a capsule-shaped compressed tablet that allows the coated product to be smaller than a capsule filled with an equivalent amount of powder. The gelatin coating facilitates swallowing and compared to unsealed capsules, gelatin-coated tablets may be more tamper-evident.

[00137] In certain embodiments, the pharmaceutical formulation may be a sublingual tablet of a cytidine analog. The sublingual tablet is intended to be dissolved beneath the tongue for absorption through the oral mucosa. The sublingual tablet may dissolve promptly and provide rapid release of the drug.

[00138] In certain embodiments, the pharmaceutical formulation is an immediate release tablet of a cytidine analog. In certain embodiments, the immediate release tablet is designed, *e.g.*, to disintegrate and release the API absent of any special rate-controlling features, such as special coatings and other techniques. In certain embodiments, the formulation is a rapidly disintegrating tablet that, *e.g.*, dissolves substantially in the mouth following administration. In certain embodiments, the pharmaceutical formulation is an extended release tablet of a cytidine analog. In certain embodiments, the extended release tablet is designed, *e.g.*, to release the API over an extended period of time and substantially in the stomach.

[00139] In certain embodiments, compressed tablets may be prepared by wet granulation. Wet granulation is a widely employed method for the production of compressed tablets, and, in particular embodiments, requires one or more the following steps: (1) weighing and blending the ingredients; (2) preparing a damp mass; (3) screening the damp mass into pellets or granules; (4) drying the granulation; (5) sizing the granulation by dry screening; (6) adding lubricant and blending; and (7) tableting by compression.

[00140] In certain embodiments, compressed tablets may be prepared by dry granulation. By the dry granulation method, the powder mixture is compacted in large pieces and subsequently broken down or sized into granules. But this method, either the active ingredient or the diluent has cohesive property. After weighing and mixing the ingredients, the powder mixture may be slugged or compressed into large flat tablets or pellets. The slugs then are broken up by hand or by a mill and passed through a screen of desired mesh for sizing. Lubricant is added in the usual manner, and tablets are prepared by compression.

Alternatively, instead of slugging, powder compactors may be used to increase the density of a powder by pressing it between high-pressure rollers. The compressed material then is broken up, sized, and lubricated, and tablets are prepared by compression in the usual manner. The roller compaction method is often preferred over slugging. Binding agents used in roller compaction formulations include methylcellulose or hydroxyl-methylcellulose and can produce good tablet hardness and friability.

[00141] In certain embodiments, compressed tablets may be prepared by direct compression. Some granular chemicals possess free flowing and cohesive properties that enable them to be compressed directly in a tablet machine without the need of wet or dry granulation. For chemicals that do not possess this quality, special pharmaceutical excipients may be used which impart the necessary qualities for the production of tablets by direct compression. Particular tableting excipients include, *e.g.*: fillers, such as spray-dried lactose, micro-crystals of alpha-monohydrate lactose, sucrose-invert sugar-corn starch mixtures, micro-crystalline cellulose, crystalline maltose, and di-calcium phosphate; disintegrating agents, such as direct-compression starch, sodium carboxymethyl starch, cross-linked carboxymethylcellulose fibers, and cross-linked polyvinylpyrrolidone; lubricants, such as magnesium searate and talc; and glidants, such as fumed silicon dioxide.

[00142] In certain embodiments, tablets provided herein may be prepared by molding. The base for molded tablets is generally a mixture of finely powdered lactose with or without a portion of powdered sucrose. In preparing the fill, the drug is mixed uniformly with the base by geometric dilution. The powder mixture may be wetted with a mixture of water and alcohol sufficient only to dampen the powder so that it may be compacted. The solvent action of the water on a portion of the lactose/sucrose base effects the biding of the powder mixture upon drying. The alcohol portion hastens the drying process.

[00143] In certain embodiments, the pharmaceutical formulations provided herein contain the cytidine analog and, optionally, one or more excipients to form a "drug core." Optional excipients include, *e.g.*, diluents (bulking agents), lubricants, disintegrants, fillers, stabilizers, surfactants, preservatives, coloring agents, flavoring agents, binding agents, excipient supports, glidants, permeation enhancement excipients, plasticizers and the like, *e.g.*, as known in the art. It will be understood by those in the art that some substances serve more than one purpose in a pharmaceutical composition. For instance, some substances are binders that help hold a tablet together after compression, yet are also disintegrants that help break the tablet apart once it reaches the target delivery site. Selection of excipients and amounts to

use may be readily determined by the formulation scientist based upon experience and consideration of standard procedures and reference works available in the art.

[00144] In certain embodiments, formulations provided herein comprise one or more binders. Binders may be used, *e.g.*, to impart cohesive qualities to a tablet, and thus ensure that the tablet remains intact after compression. Suitable binders include, but are not limited to, starch (including corn starch and pregelatinized starch), gelatin, sugars (including sucrose, glucose, dextrose and lactose), polyethylene glycol, propylene glycol, waxes, and natural and synthetic gums, *e.g.*, acacia sodium alginate, polyvinylpyrrolidone, cellulosic polymers (including hydroxypropyl cellulose, hydroxypropylmethylcellulose, methyl cellulose, ethyl cellulose, hydroxyethyl cellulose, carboxymethyl cellulose and the like), veegum, carbomer (*e.g.*, carbopol), sodium, dextrin, guar gum, hydrogenated vegetable oil, magnesium aluminum silicate, maltodextrin, polymethacrylates, povidone (*e.g.*, KOLLIDON, PLASDONE), microcrystalline cellulose, among others. Binding agents also include, *e.g.*, acacia, agar, alginic acid, cabomers, carrageenan, cellulose acetate phthalate, ceratonia, chitosan, confectioner's sugar, copovidone, dextrates, dextrin, dextrose, ethylcellulose, gelatin, glyceryl behenate, guar gum, hydroxyethyl cellulose, hydroxyethylmethyl cellulose, hydroxypropyl cellulose, hydroxypropyl starch, hypromellose, inulin, lactose, magnesium aluminum silicate, maltodextrin, maltose, methylcellulose, poloxamer, polycarbophil, polydextrose, polyethylene oxide, polymethylacrylates, povidone, sodium alginate, sodium carboxymethylcellulose, starch, pregelatinized starch, stearic acid, sucrose, and zein. The binding agent can be, relative to the drug core, in the amount of about 2% w/w of the drug core; about 4% w/w of the drug core, about 6% w/w of the drug core, about 8% w/w of the drug core, about 10% w/w of the drug core, about 12% w/w of the drug core, about 14% w/w of the drug core, about 16% w/w of the drug core, about 18% w/w of the drug core, about 20% w/w of the drug core, about 22% w/w of the drug core, about 24% w/w of the drug core, about 26% w/w of the drug core, about 28% w/w of the drug core, about 30% w/w of the drug core, about 32% w/w of the drug core, about 34% w/w of the drug core, about 36% w/w of the drug core, about 38% w/w of the drug core, about 40% w/w of the drug core, about 42% w/w of the drug core, about 44% w/w of the drug core, about 46% w/w of the drug core, about 48% w/w of the drug core, about 50% w/w of the drug core, about 52% w/w of the drug core, about 54% w/w of the drug core, about 56% w/w of the drug core, about 58% w/w of the drug core, about 60% w/w of the drug core, about 62% w/w of the drug core, about 64% w/w of the drug core, about 66% w/w of the drug core; about 68% w/w of the drug core,

about 70% w/w of the drug core, about 72% w/w of the drug core, about 74% w/w of the drug core, about 76% w/w of the drug core, about 78% w/w of the drug core, about 80% w/w of the drug core, about 82% w/w of the drug core, about 84% w/w of the drug core, about 86% w/w of the drug core, about 88% w/w of the drug core, about 90% w/w of the drug core, about 92% w/w of the drug core, about 94% w/w of the drug core, about 96% w/w of the drug core, about 98% w/w of the drug core, or more, if determined to be appropriate. In certain embodiments, a suitable amount of a particular binder is determined by one of ordinary skill in the art.

[00145] In certain embodiments, formulations provided herein comprise one or more diluents. Diluents may be used, *e.g.*, to increase bulk so that a practical size tablet is ultimately provided. Suitable diluents include dicalcium phosphate, calcium sulfate, lactose, cellulose, kaolin, mannitol, sodium chloride, dry starch, microcrystalline cellulose (*e.g.*, AVICEL), microfine cellulose, pregelitinized starch, calcium carbonate, calcium sulfate, sugar, dextrates, dextrin, dextrose, dibasic calcium phosphate dihydrate, tribasic calcium phosphate, kaolin, magnesium carbonate, magnesium oxide, maltodextrin, mannitol, polymethacrylates (*e.g.*, EUDRAGIT), potassium chloride, sodium chloride, sorbitol and talc, among others. Diluents also include, *e.g.*, ammonium alginate, calcium carbonate, calcium phosphate, calcium sulfate, cellulose acetate, compressible sugar, confectioner's sugar, dextrates, dextrin, dextrose, erythritol, ethylcellulose, fructose, fumaric acid, glyceryl palmitostearate, isomalt, kaolin, lacitol, lactose, mannitol, magnesium carbonate, magnesium oxide, maltodextrin, maltose, medium-chain triglycerides, microcrystalline cellulose, microcrystalline silicified cellulose, powered cellulose, polydextrose, polymethylacrylates, simethicone, sodium alginate, sodium chloride, sorbitol, starch, pregelatinized starch, sucrose, sulfobutylether-β-cyclodextrin, talc, tragacanth, trehalose, and xylitol. Diluents may be used in amounts calculated to obtain a desired volume for a tablet or capsule; in certain embodiments, a diluent is used in an amount of about 5% or more, about 10% or more, about 15% or more, about 20% or more, about 22% or more, about 24% or more, about 26% or more, about 28% or more, about 30% or more, about 32% or more, about 34% or more, about 36% or more, about 38% or more, about 40% or more, about 42% or more, about 44% or more, about 46% or more, about 48% or more, about 50% or more, about 52% or more, about 54% or more, about 56% or more, about 58% or more, about 60% or more, about 62% or more, about 64% or more, about 68% or more, about 70% ore more, about 72% or more, about 74% or more, about 76% or more, about 78% or more, about 80% or more, about 85%

or more, about 90% or more, or about 95% or more, weight/weight, of a drug core; between about 10% and about 90% w/w of the drug core; between about 20% and about 80% w/w of the drug core; between about 30% and about 70% w/w of the drug core; between about 40% and about 60% w/w of the drug core. In certain embodiments, a suitable amount of a particular diluent is determined by one of ordinary skill in the art.

[00146] In certain embodiments, formulations provided herein comprise one or more lubricants. Lubricants may be used, *e.g.*, to facilitate tablet manufacture; examples of suitable lubricants include, for example, vegetable oils such as peanut oil, cottonseed oil, sesame oil, olive oil, corn oil, and oil of theobroma, glycerin, magnesium stearate, calcium stearate, and stearic acid. In certain embodiments, stearates, if present, represent no more than approximately 2 weight % of the drug-containing core. Further examples of lubricants include, *e.g.*, calcium stearate, glycerin monostearate, glyceryl behenate, glyceryl palmitostearate, magnesium lauryl sulfate, magnesium stearate, myristic acid, palmitic acid, poloxamer, polyethylene glycol, potassium benzoate, sodium benzoate, sodium chloride, sodium lauryl sulfate, sodium stearyl fumarate, stearic acid, talc, and zinc stearate. In particular embodiments, the lubricant is magnesium stearate. In certain embodiments, the lubricant is present, relative to the drug core, in an amount of about 0.2% w/w of the drug core, about 0.4% w/w of the drug core, about 0.6% w/w of the drug core, about 0.8% w/w of the drug core, about 1.0% w/w of the drug core, about 1.2% w/w of the drug core, about 1.4% w/w of the drug core, about 1.6% w/w of the drug core, about 1.8% w/w of the drug core, about 2.0% w/w of the drug core, about 2.2% w/w of the drug core, about 2.4% w/w of the drug core, about 2.6% w/w of the drug core, about 2.8% w/w of the drug core, about 3.0% w/w of the drug core, about 3.5% w/w of the drug core, about 4% w/w of the drug core, about 4.5% w/w of the drug core, about 5% w/w of the drug core, about 6% w/w of the drug core, about 7% w/w of the drug core, about 8% w/w of the drug core, about 10% w/w of the drug core, about 12% w/w of the drug core, about 14% w/w of the drug core, about 16% w/w of the drug core, about 18% w/w of the drug core, about 20% w/w of the drug core, about 25% w/w of the drug core, about 30% w/w of the drug core, about 35% w/w of the drug core, about 40% w/w of the drug core, between about 0.2% and about 10% w/w of the drug core, between about 0.5% and about 5% w/w of the drug core, or between about 1% and about 3% w/w of the drug core. In certain embodiments, a suitable amount of a particular lubricant is determined by one of ordinary skill in the art.

[00147] In certain embodiments, formulations provided herein comprise one or more disintegrants. Disintegrants may be used, *e.g.*, to facilitate disintegration of the tablet, and may be, *e.g.*, starches, clays, celluloses, algins, gums or crosslinked polymers. Disintegrants also include, *e.g.*, alginic acid, carboxymethylcellulose calcium, carboxymethylcellulose sodium (*e.g.*, AC-DI-SOL, PRIMELLOSE), colloidal silicon dioxide, croscarmellose sodium, crospovidone (*e.g.*, KOLLIDON, POLYPLASDONE), guar gum, magnesium aluminum silicate, methyl cellulose, microcrystalline cellulose, polacrilin potassium, powdered cellulose, pregelatinized starch, sodium alginate, sodium starch glycolate (*e.g.*, EXPLOTAB) and starch. Additional disintegrants include, *e.g.*, calcium alginate, chitosan, sodium docusate, hydroxypropyl cellulose, and povidone. In certain embodiments, the disintegrant is, relative to the drug core, present in the amount of about 1% w/w of the drug core, about 2% w/w of the drug core, about 3% w/w of the drug core, about 4% w/w of the drug core, about 5% w/w of the drug core, about 6% w/w of the drug core, about 7% w/w of the drug core, about 8% w/w of the drug core, about 9% w/w of the drug core, about 10% w/w of the drug core, about 12% w/w of the drug core, about 14% w/w of the drug core, about 16% w/w of the drug core, about 18% w/w of the drug core, about 20% w/w of the drug core, about 22% w/w of the drug core, about 24% w/w of the drug core, about 26% w/w of the drug core, about 28% w/w of the drug core, about 30% w/w of the drug core, about 32% w/w of the drug core, greater than about 32% w/w of the drug core, between about 1% and about 10% w/w of the drug core, between about 2% and about 8% w/w of the drug core, between about 3% and about 7% w/w of the drug core, or between about 4% and about 6% w/w of the drug core. In certain embodiments, a suitable amount of a particular disintegrant is determined by one of ordinary skill in the art.

[00148] In certain embodiments, formulations provided herein comprise one or more stabilizers. Stabilizers (also called absorption enhancers) may be used, *e.g.*, to inhibit or retard drug decomposition reactions that include, by way of example, oxidative reactions. Stabilizing agents include, *e.g.*, d-Alpha-tocopheryl polyethylene glycol 1000 succinate (Vitamin E TPGS), acacia, albumin, alginic acid, aluminum stearate, ammonium alginate, ascorbic acid, ascorbyl palmitate, bentonite, butylated hydroxytoluene, calcium alginate, calcium stearate, calcium carboxymethylcellulose, carrageenan, ceratonia, colloidal silicon dioxide, cyclodextrins, diethanolamine, edetates, ethylcellulose, ethyleneglycol palmitostearate, glycerin monostearate, guar gum, hydroxypropyl cellulose, hypromellose, invert sugar, lecithin, magnesium aluminum silicate, monoethanolamine, pectin, poloxamer,

polyvinyl alcohol, potassium alginate, potassium polacrilin, povidone, propyl gallate, propylene glycol, propylene glycol alginate, raffinose, sodium acetate, sodium alginate, sodium borate, sodium carboxymethyl cellulose, sodium stearyl fumarate, sorbitol, stearyl alcohol, sufobutyl-b-cyclodextrin, trehalose, white wax, xanthan gum, xylitol, yellow wax, and zinc acetate. In certain embodiments, the stabilizer is, relative to the drug core, present in the amount of about 1% w/w of the drug core, about 2% w/w of the drug core, about 3% w/w of the drug core, about 4% w/w of the drug core, about 5% w/w of the drug core, about 6% w/w of the drug core, about 7% w/w of the drug core, about 8% w/w of the drug core, about 9% w/w of the drug core, about 10% w/w of the drug core, about 12% w/w of the drug core, about 14% w/w of the drug core, about 16% w/w of the drug core, about 18% w/w of the drug core, about 20% w/w of the drug core, about 22% w/w of the drug core, about 24% w/w of the drug core, about 26% w/w of the drug core, about 28% w/w of the drug core, about 30% w/w of the drug core, about 32% w/w of the drug core, between about 1% and about 10% w/w of the drug core, between about 2% and about 8% w/w of the drug core, between about 3% and about 7% w/w of the drug core, or between about 4% and about 6% w/w of the drug core. In certain embodiments, a suitable amount of a particular stabilizer is determined by one of ordinary skill in the art.

[00149] In certain embodiments, formulations provided herein comprise one or more glidants. Glidants may be used, *e.g.*, to improve the flow properties of a powder composition or granulate or to improve the accuracy of dosing. Excipients that may function as glidants include, *e.g.*, colloidal silicon dioxide, magnesium trisilicate, powdered cellulose, starch, tribasic calcium phosphate, calcium silicate, powdered cellulose, colloidal silicon dioxide, magnesium silicate, magnesium trisilicate, silicon dioxide, starch, tribasic calcium phosphate, and talc. In certain embodiments, the glidant is, relative to the drug core, present in the amount of less than about 1% w/w of the drug core, about 1% w/w of the drug core, about 2% w/w of the drug core, about 3% w/w of the drug core, about 4% w/w of the drug core, about 5% w/w of the drug core, about 6% w/w of the drug core, about 7% w/w of the drug core, about 8% w/w of the drug core, about 9% w/w of the drug core, about 10% w/w of the drug core, about 12% w/w of the drug core, about 14% w/w of the drug core, about 16% w/w of the drug core, about 18% w/w of the drug core, about 20% w/w of the drug core, about 22% w/w of the drug core, about 24% w/w of the drug core, about 26% w/w of the drug core, about 28% w/w of the drug core, about 30% w/w of the drug core, about 32% w/w of the drug core, between about 1% and about 10% w/w of the drug core, between about 2% and

about 8% w/w of the drug core, between about 3% and about 7% w/w of the drug core, or between about 4% and about 6% w/w of the drug core. In certain embodiments, a suitable amount of a particular glidant is determined by one of ordinary skill in the art.

[00150] In certain embodiments, formulations provided herein comprise one or more permeation enhancers (also called, *e.g.*, permeability enhancers). In certain embodiments, the permeation enhancer enhances the uptake of a cytidine analog through the gastrointestinal wall (*e.g.*, the stomach). In certain embodiments, the permeation enhancer alters the rate and/or amount of the cytidine analog that enters the bloodstream. In particular embodiments, d-alpha-tocopheryl polyethylene glycol-1000 succinate (Vitamin E TPGS) is used as a permeation enhancer. In particular embodiments, one or more other suitable permeation enhancers are used, including, *e.g.*, any permeation enhancer known in the art. Specific examples of suitable permeation enhancers include, *e.g.*, those listed below:

Product name	**Chemical Name**	**Example of Supplier**
Pluronic F 127	Poloxamer F 127	Sigma
Lutrol F 68	Poloxamer 188	BASF
Carbopol 934-P	Carbomer 934-P	Spectrum Chemical
Tween 80	Polysorbate 80	Sigma
Chitosan	Chitosan Low Mol Wt	Aldrich
Capric acid/Na cap	Sodium Decanoate	Sigma
Lauric acid/Na laur	Sodium Dodecanoate	Sigma
Disodium EDTA	Ethylenediamine tetraacetic acid disodium dihydrate	Sigma
Propylene glycol	1, 2 Propanediol	Sigma
CM Cellulose	Carboxymethyl Cellulose	Sigma
Labrasol	Caprylocaproyl macrogol-8 glycerides	Gattefosse
N,N- Dimethylacetamide	(minimum 99%)	Sigma
Vitamin E TPGS	d-Alpha-Tocopheryl Polyethylene Glycol-1000 Succinate	Eastman
Solutol HS 15	Polyethylene glycol 660 12-hydroxystearate	BASF
Labrafil M 1944 CS (2)	Oleyl Macrogolglyerides	Gattefosse

[00151] Other potential permeation enhancers include, *e.g.*, alcohols, dimethyl sulfoxide, glyceryl monooleate, glycofurol, isopropyl myristate, isopropyl palmitate, lanolin, linoleic acid, myristic acid, oleic acid, oleyl alcohol, palmitic acid, polyoxyethylene alkyl ethers, 2-pyrrolidone, sodium lauryl sulfate, and thymol.

[00152] In certain embodiments, the permeation enhancer is present in the formulation in an amount by weight, relative to the total weight of the formulation, of about 0.1%, about

0.2%, about 0.3%, about 0.4%, about 0.5%, about 0.6%, about 0.7%, about 0.8%, about 0.9%, about 1%, about 1.1%, about 1.2%, about 1.3%, about 1.4%, about 1.5%, about 1.6%, about 1.7%, about 1.8%, about 1.9%, about 2%, about 2.1%, about 2.2%, about 2.3%, about 2.4%, about 2.5%, about 2.6%, about 2.7%, about 2.8%, about 2.9%, about 3%, about 3.1%, about 3.2%, about 3.3%, about 3.4%, about 3.5%, about 3.6%, about 3.7%, about 3.8%, about 3.9%, about 4%, about 4.1% about 4.2%, about 4.3%, about 4.4%, about 4.5%, about 4.6%, about 4.7%, about 4.8%, about 4.9%, about 5%, about 5.1% about 5.2%, about 5.3%, about 5.4%, about 5.5%, about 5.6%, about 5.7%, about 5.8%, about 5.9%, about 6%, about 6.1% about 6.2%, about 6.3%, about 6.4%, about 6.5%, about 6.6%, about 6.7%, about 6.8%, about 6.9%, about 7%, about 7.1% about 7.2%, about 7.3%, about 7.4%, about 7.5%, about 7.6%, about 7.7%, about 7.8%, about 7.9%, about 8%, about 8.1% about 8.2%, about 8.3%, about 8.4%, about 8.5%, about 8.6%, about 8.7%, about 8.8%, about 8.9%, about 9%, about 9.1% about 9.2%, about 9.3%, about 9.4%, about 9.5%, about 9.6%, about 9.7%, about 9.8%, about 9.9%, about 10%, greater than about 10%, greater than about 12%, greater than about 14%, greater than about 16%, greater than about 18%, greater than about 20%, greater than about 25%, greater than about 30%, greater than about 35%, greater than about 40%, greater than about 45%, or greater than about 50%. In certain embodiments, the appropriate amount of a suitable permeation enhancer provided herein is determined by one of skill in the art.

[00153] Without intending to be limited to any particular theory, the permeation enhancers provided herein may function by, *inter alia*, facilitating (*e.g.*, increasing the rate or extent of) the transport of a cytidine analog through the gastrointestinal wall. In general, movement through the gastrointestinal wall may occur by, *e.g.*: passive diffusion, such as the movement of drug across a membrane in a manner driven solely by the concentration gradient; carrier-mediated diffusion, such as the movement of drug across a cell membrane via a specialized transport system embedded in the cell membrane; paracellular diffusion, such as the movement of a drug across a membrane by going between, rather than through, two cells; and transcellular diffusion, such as the movement of a drug across the cell. Additionally, there are numerous cellular proteins capable of preventing intracellular accumulation of drugs by pumping out drug that enters the cell. These are sometimes called efflux pumps. One such efflux pump is that involving p-glycoprotein, which is present in many different tissues in the body (*e.g.*, intestine, placental membrane, blood-brain barrier). Permeation enhancers can function by, *inter alia*, facilitating any of the processes mentioned above (such as by

increasing fluidity of membranes, opening tight junctions between cells, and/or inhibiting efflux, among others).

[00154] In certain embodiments, the compositions provided herein comprising a cytidine analog, *e.g.*, 5-azacytidine, are essentially free of a cytidine deaminase inhibitor (*e.g.*, do not comprise a cytidine deaminase inhibitor). In certain embodiments, the compositions provided herein are essentially free of (*e.g.*, do not comprise) the cytidine deaminase inhibitor tetrahydrouridine (THU). Certain embodiments herein provide pharmaceutical compositions comprising a therapeutically effective amount of a cytidine analog (*e.g.*, 5-azacytidine), wherein the compositions release the cytidine analog substantially in the stomach following oral administration to a subject, and wherein the compositions are essentially free of (*e.g.*, do not comprise) a cytidine deaminase inhibitor (*e.g.*, THU). Certain embodiments herein provide pharmaceutical compositions comprising a therapeutically effective amount of a cytidine analog (*e.g.*, 5-azacytidine), wherein the compositions release the cytidine analog substantially in the stomach following oral administration to a subject, wherein the compositions are essentially free of (*e.g.*, do not comprise) a cytidine deaminase inhibitor (*e.g.*, THU), and wherein the compositions achieve a particular biological parameter provided herein (*e.g.*, a particular Cmax value, Tmax value, and/or AUC value provided herein). In particular embodiments, a composition provided herein that is essentially free of a cytidine deaminase inhibitor (*e.g.*, THU) comprises, *e.g.*, less than 200 mg, less than 150 mg, less than 100 mg, less than 50 mg, less than 25 mg, less than 10 mg, less than 5 mg, less than 1 mg, or less than 0.1 mg of the cytidine deaminase inhibitor.

4. Additional Therapeutic Agents

[00155] In particular embodiments, the cytidine analog oral formulations provided herein further comprise one, two, three, or more other pharmacologically active substances (also termed herein "additional therapeutic agents," "second active agents," or the like). In particular embodiments, the oral formulations provided herein comprise the additional therapeutic agent(s) in a therapeutically effective amount. In particular embodiments, the cytidine analog (*e.g.*, azacitidine) and the additional therapeutic agent(s) are co-formulated together in the same dosage form using methods of co-formulating active pharmaceutical ingredients, including methods disclosed herein and methods known in the art. In other embodiments, the cytidine analog and the additional therapeutic agent(s) are co-administered in separate dosage forms. It is believed that certain combinations work synergistically in the treatment of particular diseases or disorders, including, *e.g.*, types of cancer and certain

diseases and conditions associated with, or characterized by, undesired angiogenesis or abnormal cell proliferation. Cytidine analog oral dosage forms provided herein can also work to alleviate adverse effects associated with certain second active agents, and some second active agents can be used to alleviate adverse effects associated with cytidine analog oral dosage forms provided herein. In certain embodiments, the oral formulations provided herein are co-administered with one or more therapeutic agents to provide a resensitization effect in subjects in need thereof. Additional therapeutic agents can be, *e.g.*, large molecules (*e.g.*, proteins) or small molecules (*e.g.*, synthetic inorganic, organometallic, or organic molecules).

[00156] Examples of particular additional therapeutic agents useful in the compositions and methods disclosed herein include, but are not limited to, *e.g.*, cytotoxic agents, anti-metabolites, antifolates, HDAC inhibitors (*e.g.*, entinostat, also known as SNDX-275 or MS-275; or vorinostat, also known as suberoylanilide hydroxamic acid (SAHA) or *N*-hydroxy-*N'*-phenyl-octanediamide), DNA intercalating agents, DNA cross-linking agents, DNA alkylating agents, DNA cleaving agents, topoisomerase inhibitors, CDK inhibitors, JAK inhibitors, anti-angiogenic agents, Bcr-Abl inhibitors, HER2 inhibitors, EGFR inhibitors, VEGFR inhibitors, PDGFR inhibitors, HGFR inhibitors, IGFR inhibitors, c-Kit inhibitors, Ras pathway inhibitors, PI3K inhibitors, multi-targeted kinase inhibitors, mTOR inhibitors, anti-estrogens, anti-androgens, aromatase inhibitors, somatostatin analogs, ER modulators, anti-tubulin agents, vinca alkaloids, taxanes, HSP inhibitors, Smoothened antagonists, telomerase inhibitors, COX-2 inhibitors, anti-metastatic agents, immunosuppressants, biologics such as antibodies, and hormonal therapies. In particular embodiments, the co-administered therapeutic agent is an immunomodulatory compound, *e.g.*, thalidomide, lenalidomide, or pomalidomide. The co-administered agent may be dosed, *e.g.*, orally or by injection.

[00157] Other examples of additional therapeutic agents include, but are not limited to, hematopoietic growth factor, a cytokine, an anti-cancer agent, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), erythropoietin (EPO), interleukin (IL), interferon (IFN), oblimersen, melphalan, topotecan, pentoxifylline, taxotere, irinotecan, ciprofloxacin, doxorubicin, vincristine, dacarbazine, Ara-C, vinorelbine, prednisone, cyclophosphamide, bortezomib, arsenic trioxide. Such additional therapeutic agents are particularly useful in methods and compositions disclosed herein including, but not limited to, those relating to treatment of multiple myeloma.

[00158] Other examples of additional therapeutic agents include, but are not limited to, an antibody (*e.g.*, rituximab, anti-CD33), hematopoietic growth factor, cytokine, anti-cancer agent, antibiotic, cox-2 inhibitor, immunomodulatory agent, immunosuppressive agent, corticosteroid, or a pharmacologically active mutant or derivative thereof. *See, e.g.*, S. Nand *et al.*, *Leukemia and Lymphoma*, 2008, 49(11):2141-47 (describing a Phase II study involving the administration of a combination of hydroxyurea, azacitidine and low dose gemtuzumab ozogamicin to elderly patients with AML and high-risk MDS, and concluding that this combination appears to be a safe and effective regimen in the treatment of AML and high risk MDS in this group of patients). Such additional therapeutic agents are particularly useful in methods and compositions disclosed herein including, but not limited to, those relating to treatment of the diseases and disorders disclosed herein.

[00159] Examples of large molecule active agents include, but are not limited to, hematopoietic growth factors, cytokines, and monoclonal and polyclonal antibodies. Typical large molecule active agents are biological molecules, such as naturally occurring or artificially made proteins. Proteins that are particularly useful include proteins that stimulate the survival and/or proliferation of hematopoietic precursor cells and immunologically active poietic cells *in vitro* or *in vivo*. Others stimulate the division and differentiation of committed erythroid progenitors in cells *in vitro* or *in vivo*. Particular proteins include, but are not limited to: interleukins, such as IL-2 (including recombinant IL-II ("rIL2") and canarypox IL-2), IL-10, IL-12, and IL-18; interferons, such as interferon alfa-2a, interferon alfa-2b, interferon alfa-n1, interferon alfa-n3, interferon beta-I a, and interferon gamma-I b; GM-CF and GM-CSF; and EPO.

[00160] Particular proteins that can be used in the methods and compositions provided herein include, but are not limited to: filgrastim, which is sold in the United States under the trade name Neupogen® (Amgen, Thousand Oaks, CA); sargramostim, which is sold in the United States under the trade name Leukine® (Immunex, Seattle, WA); and recombinant EPO, which is sold in the United States under the trade name Epogen® (Amgen, Thousand Oaks, CA).

[00161] Recombinant and mutated forms of GM-CSF can be prepared as described in U.S. patent nos. 5,391,485; 5,393,870; and 5,229,496; all of which are incorporated herein by reference. Recombinant and mutated forms of G-CSF can be prepared as described in U.S. patent nos. 4,810,643; 4,999,291; 5,528,823; and 5,580,755; all of which are incorporated herein by reference.

[00162] Embodiments herein encompass the use of native, naturally occurring, and recombinant proteins. Particular embodiments encompass mutants and derivatives (*e.g.*, modified forms) of naturally occurring proteins that exhibit, *in vivo*, at least some of the pharmacological activity of the proteins upon which they are based. Examples of mutants include, but are not limited to, proteins that have one or more amino acid residues that differ from the corresponding residues in the naturally occurring forms of the proteins. Also encompassed by the term "mutants" are proteins that lack carbohydrate moieties normally present in their naturally occurring forms (*e.g.*, nonglycosylated forms). Examples of derivatives include, but are not limited to, pegylated derivatives and fusion proteins, such as proteins formed by fusing IgG1 or IgG3 to the protein or active portion of the protein of interest. *See, e.g.*, Penichet, M.L. and Morrison, S.L., *J. Immunol. Methods* 248:91-101 (2001).

[00163] Antibodies that can be used in combination with oral formulations disclosed herein include monoclonal and polyclonal antibodies. Examples of antibodies include, but are not limited to, trastuzumab (Herceptin®), rituximab (Rituxan®),bevacizumab (Avastin™), pertuzumab (Omnitarg™), tositumomab (Bexxar®), edrecolomab (Panorex®), and G250. Oral formulations disclosed herein can also comprise, be combined with, or used in combination with anti-TNF-α antibodies.

[00164] Large molecule active agents may be administered in the form of anti-cancer vaccines. For example, vaccines that secrete, or cause the secretion of, cytokines such as IL-2, G-CSF, and GM-CSF can be used in the methods, pharmaceutical compositions, and kits provided herein. *See, e.g.*, Emens, L.A., *et al.*, *Curr. Opinion Mol. Ther.* 3(1):77-84 (2001).

[00165] In one embodiment, the additional therapeutic agent (*e.g.*, large-molecule compound or small-molecule compound) reduces, eliminates, or prevents an adverse effect associated with the administration (*e.g.*, oral administration) of a cytidine analog provided herein. Depending on the particular cytidine analog and the disease or disorder begin treated, adverse effects can include, but are not limited to, anemia, neutropenia, febrile neutropenia, thrombocytopenia, hepatotoxicity (*e.g.*, including, but not limited to, hepatoxicity in patients with preexisting hepatic impairment), elevated serum creatinine, renal failure, renal tubular acidosis, hypokalemia, hepatic coma, nausea, vomiting, dyspepsia, abdominal pain, pyrexia, leukopenia, diarrhea, constipation, ecchymosis, petechiae, rigors, weakness, pneumonia, anxiety, insomnia, lethargy, and decrease in weight, among others known in the art to be associated with particular cytidine analogs.

[00166] Like some large molecules, many small-molecule compounds are believed to be capable of providing a synergistic effect when administered with (*e.g.*, before, after or simultaneously) a cytidine analog oral formulation disclosed herein. Examples of small molecule second active agents include, but are not limited to, anti-cancer agents, antibiotics, immunosuppressive agents, and steroids.

[00167] Examples of anti-cancer agents include, but are not limited to: acivicin; aclarubicin; acodazole hydrochloride; acronine; adozelesin; aldesleukin; altretamine; ambomycin; ametantrone acetate; amsacrine; anastrozole; anthramycin; asparaginase; asperlin; azacitidine; azetepa; azotomycin; batimastat; benzodepa; bicalutamide; bisantrene hydrochloride; bisnafide dimesylate; bizelesin; bleomycin sulfate; brequinar sodium; bropirimine; busulfan; cactinomycin; calusterone; caracemide; carbetimer; carboplatin; carmustine; carubicin hydrochloride; carzelesin; cedefingol; celecoxib (COX-2 inhibitor); chlorambucil; cirolemycin; cisplatin; cladribine; crisnatol mesylate; cyclophosphamide; cytarabine; dacarbazine; dactinomycin; daunorubicin hydrochloride; decitabine; dexormaplatin; dezaguanine; dezaguanine mesylate; diaziquone; docetaxel; doxorubicin; doxorubicin hydrochloride; droloxifene; droloxifene citrate; dromostanolone propionate; duazomycin; edatrexate; eflornithine hydrochloride; elsamitrucin; enloplatin; enpromate; epipropidine; epirubicin hydrochloride; erbulozole; esorubicin hydrochloride; estramustine; estramustine phosphate sodium; etanidazole; etoposide; etoposide phosphate; etoprine; fadrozole hydrochloride; fazarabine; fenretinide; floxuridine; fludarabine phosphate; fluorouracil; flurocitabine; fosquidone; fostriecin sodium; gemcitabine; gemcitabine hydrochloride; hydroxyurea; idarubicin hydrochloride; ifosfamide; ilmofosine; iproplatin; irinotecan; irinotecan hydrochloride; lanreotide acetate; letrozole; leuprolide acetate; liarozole hydrochloride; lometrexol sodium; lomustine; losoxantrone hydrochloride; masoprocol; maytansine; mechlorethamine hydrochloride; megestrol acetate; melengestrol acetate; melphalan; menogaril; mercaptopurine; methotrexate; methotrexate sodium; metoprine; meturedepa; mitindomide; mitocarcin; mitocromin; mitogillin; mitomalcin; mitomycin; mitosper; mitotane; mitoxantrone hydrochloride; mycophenolic acid; nocodazole; nogalamycin; ormaplatin; oxisuran; paclitaxel; pegaspargase; peliomycin; pentamustine; peplomycin sulfate; perfosfamide; pipobroman; piposulfan; piroxantrone hydrochloride; plicamycin; plomestane; porfimer sodium; porfiromycin; prednimustine; procarbazine hydrochloride; puromycin; puromycin hydrochloride; pyrazofurin; riboprine; safingol; safingol hydrochloride; semustine; simtrazene; sparfosate sodium; sparsomycin;

spirogermanium hydrochloride; spiromustine; spiroplatin; streptonigrin; streptozocin; sulofenur; talisomycin; tecogalan sodium; taxotere; tegafur; teloxantrone hydrochloride; temoporfin; teniposide; teroxirone; testolactone; thiamiprine; thioguanine; thiotepa; tiazofurin; tirapazamine; toremifene citrate; trestolone acetate; triciribine phosphate; trimetrexate; trimetrexate glucuronate; triptorelin; tubulozole hydrochloride; uracil mustard; uredepa; vapreotide; verteporfin; vinblastine sulfate; vincristine sulfate; vindesine; vindesine sulfate; vinepidine sulfate; vinglycinate sulfate; vinleurosine sulfate; vinorelbine tartrate; vinrosidine sulfate; vinzolidine sulfate; vorozole; zeniplatin; zinostatin; and zorubicin hydrochloride.

[00168] Other anti-cancer drugs include, but are not limited to: 20-epi-1,25 dihydroxyvitamin D3; 5-ethynyluracil; abiraterone; aclarubicin; acylfulvene; adecypenol; adozelesin; aldesleukin; ALL-TK antagonists; altretamine; ambamustine; amidox; amifostine; aminolevulinic acid; amrubicin; amsacrine; anagrelide; anastrozole; andrographolide; angiogenesis inhibitors; antagonist D; antagonist G; antarelix; anti-dorsalizing morphogenetic protein-1; antiandrogen, prostatic carcinoma; antiestrogen; antineoplaston; antisense oligonucleotides; aphidicolin glycinate; apoptosis gene modulators; apoptosis regulators; apurinic acid; ara-CDP-DL-PTBA; arginine deaminase; asulacrine; atamestane; atrimustine; axinastatin 1; axinastatin 2; axinastatin 3; azasetron; azatoxin; azatyrosine; baccatin III derivatives; balanol; batimastat; BCR/ABL antagonists; benzochlorins; benzoylstaurosporine; beta lactam derivatives; beta-alethine; betaclamycin B; betulinic acid; bFGF inhibitor; bicalutamide; bisantrene; bisaziridinylspermine; bisnafide; bistratene A; bizelesin; breflate; bropirimine; budotitane; buthionine sulfoximine; calcipotriol; calphostin C; camptothecin derivatives; capecitabine; carboxamide-amino-triazole; carboxyamidotriazole; CaRest M3; CARN 700; cartilage derived inhibitor; carzelesin; casein kinase inhibitors (ICOS); castanospermine; cecropin B; cetrorelix; chlorlns; chloroquinoxaline sulfonamide; cicaprost; cis-porphyrin; cladribine; clomifene analogues; clotrimazole; collismycin A; collismycin B; combretastatin A4; combretastatin analogue; conagenin; crambescidin 816; crisnatol; cryptophycin 8; cryptophycin A derivatives; curacin A; cyclopentanthraquinones; cycloplatam; cypemycin; cytarabine ocfosfate; cytolytic factor; cytostatin; dacliximab; decitabine; dehydrodidemnin B; deslorelin; dexamethasone; dexifosfamide; dexrazoxane; dexverapamil; diaziquone; didemnin B; didox; diethylnorspermine; dihydro-5-azacytidine; dihydrotaxol, 9-; dioxamycin; diphenyl spiromustine; docetaxel; docosanol; dolasetron; doxifluridine;

doxorubicin; droloxifene; dronabinol; duocarmycin SA; ebselen; ecomustine; edelfosine; edrecolomab; eflornithine; elemene; emitefur; epirubicin; epristeride; estramustine analogue; estrogen agonists; estrogen antagonists; etanidazole; etoposide phosphate; exemestane; fadrozole; fazarabine; fenretinide; filgrastim; finasteride; flavopiridol; flezelastine; fluasterone; fludarabine; fluorodaunorunicin hydrochloride; forfenimex; formestane; fostriecin; fotemustine; gadolinium texaphyrin; gallium nitrate; galocitabine; ganirelix; gelatinase inhibitors; gemcitabine; glutathione inhibitors; hepsulfam; heregulin; hexamethylene bisacetamide; hypericin; ibandronic acid; idarubicin; idoxifene; idramantone; ilmofosine; ilomastat; imatinib (*e.g.*, Gleevec®), imiquimod; immunostimulant peptides; insulin-like growth factor-1 receptor inhibitor; interferon agonists; interferons; interleukins; iobenguane; iododoxorubicin; ipomeanol, 4-; iroplact; irsogladine; isobengazole; isohomohalicondrin B; itasetron; jasplakinolide; kahalalide F; lamellarin-N triacetate; lanreotide; leinamycin; lenograstim; lentinan sulfate; leptolstatin; letrozole; leukemia inhibiting factor; leukocyte alpha interferon; leuprolide+estrogen+progesterone; leuprorelin; levamisole; liarozole; linear polyamine analogue; lipophilic disaccharide peptide; lipophilic platinum compounds; lissoclinamide 7; lobaplatin; lombricine; lometrexol; lonidamine; losoxantrone; loxoribine; lurtotecan; lutetium texaphyrin; lysofylline; lytic peptides; maitansine; mannostatin A; marimastat; masoprocol; maspin; matrilysin inhibitors; matrix metalloproteinase inhibitors; menogaril; merbarone; meterelin; methioninase; metoclopramide; MIF inhibitor; mifepristone; miltefosine; mirimostim; mitoguazone; mitolactol; mitomycin analogues; mitonafide; mitotoxin fibroblast growth factor-saporin; mitoxantrone; mofarotene; molgramostim;Erbitux, human chorionic gonadotrophin; monophosphoryl lipid A+myobacterium cell wall sk; mopidamol; mustard anticancer agent; mycaperoxide B; mycobacterial cell wall extract; myriaporone; N-acetyldinaline; N-substituted benzamides; nafarelin; nagrestip; naloxone+pentazocine; napavin; naphterpin; nartograstim; nedaplatin; nemorubicin; neridronic acid; nilutamide; nisamycin; nitric oxide modulators; nitroxide antioxidant; nitrullyn; oblimersen (Genasense®); O^6-benzylguanine; octreotide; okicenone; oligonucleotides; onapristone; ondansetron; ondansetron; oracin; oral cytokine inducer; ormaplatin; osaterone; oxaliplatin; oxaunomycin; paclitaxel; paclitaxel analogues; paclitaxel derivatives; palauamine; palmitoylrhizoxin; pamidronic acid; panaxytriol; panomifene; parabactin; pazelliptine; pegaspargase; peldesine; pentosan polysulfate sodium; pentostatin; pentrozole; perflubron; perfosfamide; perillyl alcohol; phenazinomycin; phenylacetate; phosphatase inhibitors; picibanil; pilocarpine hydrochloride;

pirarubicin; piritrexim; placetin A; placetin B; plasminogen activator inhibitor; platinum complex; platinum compounds; platinum-triamine complex; porfimer sodium; porfiromycin; prednisone; propyl bis-acridone; prostaglandin J2; proteasome inhibitors; protein A-based immune modulator; protein kinase C inhibitor; protein kinase C inhibitors, microalgal; protein tyrosine phosphatase inhibitors; purine nucleoside phosphorylase inhibitors; purpurins; pyrazoloacridine; pyridoxylated hemoglobin polyoxyethylene conjugate; raf antagonists; raltitrexed; ramosetron; ras farnesyl protein transferase inhibitors; ras inhibitors; ras-GAP inhibitor; retelliptine demethylated; rhenium Re 186 etidronate; rhizoxin; ribozymes; RII retinamide; rohitukine; romurtide; roquinimex; rubiginone B1; ruboxyl; safingol; saintopin; SarCNU; sarcophytol A; sargramostim; Sdi 1 mimetics; semustine; senescence derived inhibitor 1; sense oligonucleotides; signal transduction inhibitors; sizofiran; sobuzoxane; sodium borocaptate; sodium phenylacetate; solverol; somatomedin binding protein; sonermin; sparfosic acid; spicamycin D; spiromustine; splenopentin; spongistatin 1; squalamine; stipiamide; stromelysin inhibitors; sulfinosine; superactive vasoactive intestinal peptide antagonist; suradista; suramin; swainsonine; tallimustine; tamoxifen methiodide; tauromustine; tazarotene; tecogalan sodium; tegafur; tellurapyrylium; telomerase inhibitors; temoporfin; teniposide; tetrachlorodecaoxide; tetrazomine; thaliblastine; thiocoraline; thrombopoietin; thrombopoietin mimetic; thymalfasin; thymopoietin receptor agonist; thymotrinan; thyroid stimulating hormone; tin ethyl etiopurpurin; tirapazamine; titanocene bichloride; topsentin; toremifene; translation inhibitors; tretinoin; triacetyluridine; triciribine; trimetrexate; triptorelin; tropisetron; turosteride; tyrosine kinase inhibitors; tyrphostins; UBC inhibitors; ubenimex; urogenital sinus-derived growth inhibitory factor; urokinase receptor antagonists; vapreotide; variolin B; velaresol; veramine; verdins; verteporfin; vinorelbine; vinxaltine; vitaxin; vorozole; zanoterone; zeniplatin; zilascorb; and zinostatin stimalamer.

[00169] Specific additional therapeutic agents include, but are not limited to, oblimersen (Genasense®), remicade, docetaxel, celecoxib, melphalan, dexamethasone (Decadron®), steroids, gemcitabine, cisplatinum, temozolomide, etoposide, cyclophosphamide, temodar, carboplatin, procarbazine, gliadel, tamoxifen, topotecan, methotrexate, Arisa®, taxol, taxotere, fluorouracil, leucovorin, irinotecan, xeloda, CPT-11, interferon alpha, pegylated interferon alpha (*e.g.*, PEG INTRON-A), capecitabine, cisplatin, thiotepa, fludarabine, carboplatin, liposomal daunorubicin, cytarabine, doxetaxol, pacilitaxel, vinblastine, IL-2, GM-CSF, dacarbazine, vinorelbine, zoledronic acid, palmitronate, biaxin, busulphan,

prednisone, bisphosphonate, arsenic trioxide, vincristine, doxorubicin (Doxil®), paclitaxel, ganciclovir, adriamycin, estramustine sodium phosphate (Emcyt®), sulindac, and etoposide.

D. Methods of Use

[00170] As described herein, certain embodiments herein provide oral formulations of cytidine analogs useful in methods relating to, *e.g.*, permitting different dosing amounts and/or dosing periods; providing alternative pharmacokinetic profiles, pharmacodynamic profiles, and/or safety profiles; permitting the evaluation of long-term and/or maintenance therapies; providing treatment regimens that maximize demethylation and/or gene re-expression; providing treatment regimens that prolong continuous demethylation; providing new indications for cytidine analogs; and/or providing other potential advantageous benefits.

[00171] Provided herein are methods of treating patho-physiological conditions manifested by abnormal cell proliferation, such as, for example, cancer, including hematological disorders and solid tumors, by orally administering a pharmaceutical formulation comprising a cytidine analog, such as, for example, 5-azacytidine, wherein the formulation releases the cytidine analog substantially in the stomach. Other embodiments herein provide methods of treating immune disorders. In particular embodiments, the methods provided herein involve oral administering a formulation that effects an immediate release of the cytidine analog. In certain embodiments, the cytidine analog and one or more therapeutic agents are co-administered to subjects to yield a synergistic therapeutic effect. The co-administered agent may be a cancer therapeutic agent dosed orally or by injection.

[00172] In certain embodiments, methods provided herein for treating disorders related to abnormal cell proliferation comprise orally administering a formulation comprising a therapeutically effective amount of a cytidine analog. Particular therapeutic indications relating to the methods provided herein are disclosed herein. In certain embodiments, the therapeutically effective amount of the cytidine analog in the pharmaceutical formulation is an amount as disclosed herein. In certain embodiments, the precise therapeutically effective amount of the cytidine analog in the pharmaceutical formulation will vary depending on, *e.g.*, the age, weight, disease and/or condition of the subject.

[00173] In particular embodiments, the disorders related to abnormal cell proliferation include, but are not limited to, MDS, AML, ALL, CML, leukemia, chronic lymphocytic leukemia (CLL), lymphoma (including non-Hodgkin's lymphoma (NHL) and Hodgkin's lymphoma), multiple myeloma (MM), sarcoma, melanoma, carcinoma, adenocarcinoma, chordoma, breast cancer, colorectal cancer, ovarian cancer, lung cancer (*e.g.*, non-small-cell

lung cancer and small-cell lung cancer), testicular cancer, renal cancer, pancreatic cancer, bone cancer, gastric cancer, head and neck cancer, and prostate cancer. In particular embodiment, the disorder related to abnormal cell proliferation is MDS. In particular embodiments, the disorder related to abnormal cell proliferation is AML.

[00174] In certain embodiments, methods provided herein for treating disorders of abnormal cell proliferation comprise administering a cytidine analog using at least two of IV, SC and oral administration methods. For example, particular embodiments herein provide administering an initial treatment cycle of a cytidine analog, such as, for example, 5-azacytidine, administered either SC or IV, followed by subsequent orally administered treatment cycles of the cytidine analog. In certain embodiments, treatment cycles comprise multiple doses administered to a subject in need thereof over multiple days (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or greater than 14 days), optionally followed by treatment dosing holidays (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or greater than 14 days). Particular embodiments herein provide a treatment schedule comprising SC and/or IV administration for one, two, three, four, five, or more initial cycles, followed by oral administration for subsequent cycles. For example, particular embodiments herein provide a treatment schedule comprising SC administration for cycle 1, followed by oral administration for subsequent cycles. Suitable dosage ranges and amounts for the methods provided herein are provided throughout the specification. For example, in certain embodiments, the SC dose is about 75 mg/m^2. In certain embodiments, the oral dose is about 60 mg, about 80 mg, about 120 mg, about 180 mg, about 240 mg, about 300 mg, about 360 mg, about 480 mg, or greater than about 480 mg. In certain embodiments, oral doses are calculated to achieve 80%, 100%, or 120% of SC AUC.

[00175] In certain embodiments, methods of treating disorders of abnormal cell proliferation comprises orally administering a formulation comprising a cytidine analog (*e.g.*, 5-azacytidine) as single or multiple daily doses. In particular embodiments, the formulation(s) comprising the cytidine analog is/are orally administered once per day, twice per day, three times per day, four times per day, or more than four times per day. For example, in certain embodiments, the formulation comprising the cytidine analog is administered using a treatment cycle comprising administration of about 200 mg, about 300 mg, about 400 mg, about 500 mg, about 600 mg, about 700 mg, about 800 mg, about 900 mg, or about 1,000 mg of the cytidine analog once, twice, three, or four times per day for 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 days. In

certain embodiments, the method of treating comprises continuous low-dose administration. In certain embodiments, the formulation comprising the cytidine analog is administered using a treatment cycle comprising administration of about 300 mg of the cytidine analog twice per day for 7 days. In certain embodiments, the formulation comprising the cytidine analog is administered using a treatment cycle comprising administration of about 300 mg of the cytidine analog twice per day for 14 days. In certain embodiments, the formulation comprising the cytidine analog is administered using a treatment cycle comprising administration of about 300 mg of the cytidine analog three times per day for 7 days. In certain embodiments, the formulation comprising the cytidine analog is administered using a treatment cycle comprising administration of about 300 mg of the cytidine analog three times per day for 14 days. In certain embodiments, methods provided herein comprise administering a formulation comprising a cytidine analog using one or more of the cycles provided herein, and repeating one or more of the cycles for a period of, *e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or greater than 12 months.

[00176] In certain embodiments, methods herein comprise administering particular oral formulations provided herein to, *e.g.*, overcome limitations associated with IV or SC administration of cytidine analogs. For example, IV or SC administration may limit the ability to deliver a cytidine analog for longer periods of time on a regular basis, thereby potentially limiting the maximal efficacy of the cytidine analog. Due to the difficulties of complying with the rigors of a prolonged IV or SC dosing schedule, prolonged SC or IV exposure to a cytidine analog may cause subjects (*e.g.*, subjects with multiple cytopenias) to discontinue from the regimen. *See, e.g.*, Lyons, R.M., *et al.*, Hematologic Response to Three Alternative Dosing Schedules of Azacitidine in Patients With Myelodysplastic Syndromes, *J. Clin. Oncol.* (2009) (DOI:10.1200/ JCO.2008.17.1058), which is incorporated by reference herein in its entirety. Accordingly, in certain embodiments, methods provided herein comprise administering an oral formulation provided herein to overcome these or other limitations associated with SC or IV cytidine analog administration. For example, in certain embodiments, methods provided herein comprise administering daily to a subject an oral formulation provided herein for 7 or more, 8 or more, 9 or more, 10 or more, 11 or more, 12 or more, 13 or more, 14 or more, 15 or more, 16 or more, 17 or more, 18 or more, 19 or more, 20 or more, or 21 or more days.

[00177] Certain embodiments herein provide methods comprising administering oral formulations of cytidine analogs provided herein comprising delivering the cytidine analog

(*e.g.*, azacitidine) at a lower dose over a more prolonged period of time, as compared to IV or SC administration. In particular embodiments, such methods comprise managing dose-related cytopenias (including, *e.g.*, dose-related cytopenias associated with azacitidine) by administering an oral formulation provided herein. In certain embodiments, methods provided herein comprise administering an oral formulation provided herein to achieve an improved safety profile as compared to an IV or SC dose comprising the same cytidine analog.

[00178] As described herein, certain embodiments provide methods for improved treatment of particular diseases or disorders (*e.g.*, treatment of solid tumors) by administering an oral formulation provided herein, as compared to IV or SC administration of the cytidine analog. In particular embodiments, certain methods herein provide administering oral formulations provided herein at lower doses for more prolonged periods of time, leading to improved demethylation. For example, certain methods provided herein comprise administering an oral formulation provided herein to treat a solid tumor while avoiding certain dose-limiting-toxicity-related side effects associated with dosing the cytidine analog via SC or IV administration. An example of certain toxicity-related drawbacks associated with administration of a cytidine analog are described, *e.g.*, in K. Appleton *et al.*, *J. Clin. Oncol.*, Vol. 25(29):4603-4609 (2007), which is incorporated by reference herein in its entirety.

[00179] Particular embodiments herein provide methods for treating a subject having a disease or disorder provided herein by orally administering a pharmaceutical composition provided herein, wherein the treatment results in improved survival of the subject. In certain embodiments, the improved survival is measured as compared to one or more conventional care regimens. Particular embodiments herein provide methods for treating a subject having a disease or disorder provided herein by orally administering a pharmaceutical composition provided herein, wherein the treatment provides improved effectiveness. In particular embodiments, the improved effectiveness is measured using one or more endpoints for cancer clinical trials, as recommended by the U.S. Food and Drug Administration (FDA). For example, FDA provides Gudiance for Industry on Clinical Trial Endpoints for the Approval of Cancer Drugs and Biologics (http://www.fda.gov/CbER/gdlns/clintrialend.htm). The FDA endpoints include, but are not limited to, Overall Survival, Endpoints Based on Tumor Assessments such as (i) Disease-Free Survival (ii) Objective Response Rate, (iii) Time to Progression and Progression-Free Survival and (iv) Time-to-Treatment Failure. Endpoints

Involving Symptom Endpoints may include Specific Symptom Endpoints such as (i) Time to progression of cancer symptoms and (ii) A composite symptom endpoint. Biomarkers assayed from blood or body fluids may also be useful to determine the management of the disease.

[00180] In certain embodiments, the methods of treating disorders of abnormal cell proliferation comprise orally administering a formulation of a cytidine analog with food. In certain embodiments, the methods of treating disorders of abnormal cell proliferation comprise orally administering a formulation of a cytidine analog without food. In certain embodiments, pharmacological parameters (*e.g.*, Cmax, Tmax) depend on the fed state of the subject. In certain embodiments, the formulation of the cytidine analog is administered sublingually.

[00181] In certain embodiments, the cytidine analog, *e.g.*, 5-azacytidine, is not co-administered with a cytidine deaminase inhibitor. In certain embodiments, the oral formulation comprising a cytidine analog as provided herein is not co-administered with THU. Certain embodiments herein provide methods of treating a disease or disorder provided herein (*e.g.*, a disease associated with abnormal cell proliferation) comprising orally administering a cytidine analog provided herein (*e.g.*, 5-azacytidine) for release substantially in the stomach, wherein the methods achieve a particular biological parameter provided herein (*e.g.*, a particular Cmax value, Tmax value, and/or AUC value provided herein), and wherein the methods comprise not co-administering a cytidine deaminase inhibitor with the cytidine analog. Certain embodiments herein provide methods of treating a disease or disorder provided herein (*e.g.*, a disease associated with abnormal cell proliferation) comprising orally administering a cytidine analog provided herein (*e.g.*, 5-azacytidine) for release substantially in the stomach, wherein the methods avoid adverse effects associated with administering a cytidine deaminase inhibitor (*e.g.*, THU) by not co-administering the cytidine deaminase inhibitor with the cytidine analog. In particular embodiments, a cytidine deaminase inhibitor (*e.g.*, THU) is co-administered with the cytidine analog in an amount of, *e.g.*, less than about 500 mg/d, less than about 200 mg/d, less than about 150 mg/d, less than about 100 mg/d, less than about 50 mg/d, less than about 25 mg/d, less than about 10 mg/d, less than about 5 mg/d, less than about 1 mg/d, or less than about 0.1 mg/d.

[00182] In certain embodiments, methods provided herein comprise treating a disorder provided herein, including a hematologic disorder, by administering an oral dosage form comprising a cytidine analog to a subject in need thereof. In particular embodiments, oral

dosage forms provided herein comprising 5-azacytidine are used to treat subjects having hematologic disorders. Hematologic disorders include, *e.g.*, abnormal growth of blood cells which can lead to dysplastic changes in blood cells and hematologic malignancies such as various leukemias. Examples of hematologic disorders include, but are not limited to, acute myeloid leukemia (AML), acute promyelocytic leukemia (APML), acute lymphoblastic leukemia (ALL), chronic myelogenous leukemia (CML), chronic lymphocytic leukemia (CLL), myelodysplastic syndromes (MDS), and sickle cell anemia, among others. Other disorders that can be treated using the methods provided herein include, *e.g.*, multiple myeloma (MM) and non-Hodgkin's lymphoma (NHL).

[00183] In certain embodiments, methods provided herein comprise treating AML by administering an oral dosage form comprising a cytidine analog to a subject in need thereof. AML is the most common type of acute leukemia that occurs in adults. Several inherited genetic disorders and immunodeficiency states are associated with an increased risk of AML. These include disorders with defects in DNA stability, leading to random chromosomal breakage, such as Bloom's syndrome, Fanconi's anemia, Li-Fraumeni kindreds, ataxia-telangiectasia, and X-linked agammaglobulinemia.

[00184] In certain embodiments, methods provided herein comprise treating APML by administering an oral dosage form comprising a cytidine analog to a subject in need thereof. APML represents a distinct subgroup of AML. This subtype is characterized by promyelocytic blasts containing the 15;17 chromosomal translocation. This translocation leads to the generation of the fusion transcript comprised of the retinoic acid receptor and a sequence PML.

[00185] In certain embodiments, methods provided herein comprise treating ALL by administering an oral dosage form comprising a cytidine analog to a subject in need thereof. ALL is a heterogenerous disease with distinct clinical features displayed by various subtypes. Reoccurring cytogenetic abnormalities have been demonstrated in ALL. The most common cytogenetic abnormality is the 9;22 translocation. The resultant Philadelphia chromosome represents poor prognosis of the subject.

[00186] In certain embodiments, methods provided herein comprise treating CML by administering an oral dosage form comprising a cytidine analog to a subject in need thereof. CML is a clonal myeloproliferative disorder of a pluripotent stem cell. CML is characterized by a specific chromosomal abnormality involving the translocation of chromosomes 9 and

22, creating the Philadelphia chromosome. Ionizing radiation is associated with the development of CML.

[00187] In certain embodiments, methods provided herein comprise treating MDS by administering an oral dosage form comprising a cytidine analog to a subject in need thereof. In certain embodiments, MDS includes one or more of the following myelodysplastic syndrome subtypes: refractory anemia, refractory anemia with ringed sideroblasts (if accompanied by neutropenia or thrombocytopenia or requiring transfusions), refractory anemia with excess blasts, refractory anemia with excess blasts in transformation, and chronic myelomonocytic leukemia. In certain embodiments, the MDS is higher-risk MDS. In certain embodiments, the methods provided herein comprise administering an oral dosage form comprising a cytidine analog to a subject in need thereof to increase the survival (*e.g.*, prolong the life) of a subject with MDS.

[00188] In certain embodiments, methods provided herein comprise treating NHL by administering an oral dosage form comprising a cytidine analog to a subject in need thereof. Non-Hodgkin's Lymphomas (NHL) represent a heterogeneous group of malignancies of the lymphoid system. According to the WHO classification of hematological and lymphoid tumors, these diseases are classified as B-cell and T-cell neoplasms. B-cell lymphomas account for about 90% of all lymphomas, and the two most common histological disease entities are follicular lymphoma and diffuse large B-cell lymphoma. Approximately 55,000 to 60,000 new cases of NHL are diagnosed annually in the U.S. *See, e.g.*, Ansell, S.M., *et al.*, *Mayo Clin. Proc.*, 2005, 80(8):1087-97.

[00189] In certain embodiments, methods provided herein comprise treating MM by administering an oral dosage form comprising a cytidine analog to a subject in need thereof. Multiple myeloma is one of the most commonly diagnosed hematologic malignancies. In 2007, in the U.S. alone, there were roughly 20,000 new MM cases and 10,000 deaths due to MM. The disease is characterized by, *inter alia*, an accumulation of malignant plasma cells in the bone marrow, which can lead to the overproduction of an immunoglobulin, *e.g.*, a monoclonal immunoglobulin G or A. These immunoglobulins, also known as paraproteins, can be detected in the urine and blood of patients with MM. Consequences of MM include anemia, the development of destructive bony lesions, and renal insufficiency. *See, e.g.*, Rao, K.V., *American Journal of Health-System Pharmacy*, 2007, 64(17):1799-1807.

[00190] In certain embodiments, methods provided herein comprise treating CLL by administering an oral dosage form comprising a cytidine analog to a subject in need thereof.

Chronic lymphocytic lymphoma (CLL) is a malignancy of mature B lymphocytes and is the most prevalent lymphoid malignancy in the U.S. The WHO classification of B lymphocytic neoplasms groups B cell malignancies according to the presumed normal counterpart of the malignant cells. CLL is diagnosed by immunophenotype analysis of lymphocytes from the blood, bone marrow, or lymph nodes. *See, e.g.*, Zent, C.S., *et al.*, *Current Oncology Reports*, 2007, 9:345-52.

[00191] Certain embodiments herein provide methods for delivering a cytidine analog to a subject comprising administering to the subject in need thereof an oral formulation comprising a cytidine analog. In particular embodiments, oral formulations comprise (1) a therapeutically effective amount of a cytidine analog; and (2) an optional drug release controlling component capable of releasing the cytidine analog substantially in the stomach after a subject ingests the oral formulation comprising the cytidine analog. Certain embodiments herein provide a method for enhancing the oral bioavailability of a cytidine analog in a subject. Certain embodiments herein provide a method of increasing the oral bioavailability of a cytidine analog comprising orally administering a pharmaceutical composition provided herein. In certain methods provided herein, a pharmaceutical composition provided herein is orally administered to a subject, contacts the biological fluids of the subject's body, and is absorbed in the upper gastrointestinal tract, such as, for example, substantially in the stomach.

[00192] Certain embodiments herein provide a method of achieving a particular exposure value provided herein by administering an oral formulation comprising a cytidine analog (*e.g.*, 5-azacytidine) provided herein. Certain embodiments herein provide a method of achieving a particular oral bioavailability value provided herein by administering an oral formulation comprising a cytidine analog (*e.g.*, 5-azacytidine) provided herein. Certain embodiments herein provide a method of achieving a particular AUC value provided herein by administering an oral formulation comprising a cytidine analog (*e.g.*, 5-azacytidine) provided herein. Certain embodiments herein provide a method of achieving a particular Cmax value provided herein by administering an oral formulation comprising a cytidine analog (*e.g.*, 5-azacytidine) provided herein. Certain embodiments herein provide a method of achieving a particular Tmax value provided herein by administering an oral formulation comprising a cytidine analog (*e.g.*, 5-azacytidine) provided herein.

[00193] Certain embodiments herein provide methods of treating a condition involving undesirable or uncontrolled cell proliferation by administering an oral formulation

comprising a cytidine analog (*e.g.*, 5-azacytidine) as provided herein. Such conditions include, *e.g.*, benign tumors, various types of cancers such as primary tumors and tumor metastasis, hematological disorders (*e.g.* leukemia, myelodysplastic syndrome and sickle cell anemia), restenosis (*e.g.* coronary, carotid, and cerebral lesions), abnormal stimulation of endothelial cells (arteriosclerosis), insults to body tissue due to surgery, abnormal wound healing, abnormal angiogenesis, diseases that produce fibrosis of tissue, repetitive motion disorders, disorders of tissues that are not highly vascularized, and proliferative responses associated with organ transplants.

[00194] In certain embodiments, cells in a benign tumor retain their differentiated features and do not divide in a completely uncontrolled manner. A benign tumor may be localized and/or nonmetastatic. Specific types of benign tumors that can be treated using the methods, compositions, and formulations provided herein include, *e.g.*, hemangiomas, hepatocellular adenoma, cavernous hemangioma, focal nodular hyperplasia, acoustic neuromas, neurofibroma, bile duct adenoma, bile duct cystanoma, fibroma, lipomas, leiomyomas, mesotheliomas, teratomas, myxomas, nodular regenerative hyperplasia, trachomas and pyogenic granulomas.

[00195] In certain embodiments, cells in a malignant tumor become undifferentiated, do not respond to the body's growth control signals, and/or multiply in an uncontrolled manner. The malignant tumor may be invasive and capable of spreading to distant sites (metastasizing). Malignant tumors may be divided into two categories: primary and secondary. Primary tumors arise directly from the tissue in which they are found. A secondary tumor, or metastasis, is a tumor which is originated elsewhere in the body but has now spread to a distant organ. The common routes for metastasis are direct growth into adjacent structures, spread through the vascular or lymphatic systems, and tracking along tissue planes and body spaces (peritoneal fluid, cerebrospinal fluid, etc.).

[00196] Methylation can lead to the silencing of genes critical to cellular control (*i.e.*, epigenetic gene silencing), and can be an early event in the development of malignant tumors including, *e.g.*, colorectal cancer or lung cancer. *See, e.g.*, M.V. Brock *et al.*, *N. Engl. J. Med.*, 2008, 358(11):1118-28; P.M. Das *et al.*, *Mol. Cancer*, 2006, 5(28); G. Gifford *et al.*, *Clin. Cancer Res.*, 2004, 10:4420-26; J.G. Herman *et al.*, *N. Engl. J. Med.*, 2003, 349:2042-54; A.M. Jubb *et al.*, *J. Pathology*, 2001, 195:111-34. Accordingly, in certain embodiments, methods herein provide using oral formulations provided herein to prevent or reverse epigenetic gene silencing, *e.g.*, by reversing abnormal DNA methylation. In specific

embodiments, oral formulations provided herein are used for early intervention to prevent the development of cancer in patients at risk of developing cancer, *e.g.*, familial polyposis or lung cancer, wherein a cause of the cancer is epigenetic gene silencing. In particular embodiments, such early intervention would be impractical by means other than oral administration (*e.g.*, IV or SC administration). In specific embodiments, oral formulations provided herein are used for early intervention to prevent the recurrence of cancer in patients at risk for early relapse, *e.g.*, colorectal cancer or non-small-cell lung cancer. In certain embodiments, the early intervention is achieved via prolonged oral dosing schedules, using formulations and/or methods as described herein. Certain embodiments provide methods for administering oral formulations provided herein to reverse the effect of gene silencing, *e.g.*, in patients at risk of gene silencing due to epigenetic changes. In particular embodiments, methods provided herein further comprise administering an HDAC inhibitor compound (*e.g.*, to restore chromatin to a transcriptionally active configuration after reversing abnormal DNA methylation). In particular embodiments, the HDAC inhibitor compound is entinostat (SNDX-275; formerly MS-275), an oral HDAC inhibitor that acts synergistically with targeted therapies and is selective for cancer-relevant HDAC isoforms 1, 2, and 3. In particular embodiments, a synergistic effect is achieved by co-administering 5-azacytidine and an HDAC inhibitor (*e.g.*, etinostat) for the treatment of solid tumors (*e.g.*, NSCLC) or hematological malignancies (*e.g.*, MDS, CMMoL, or AML).

[00197] In certain embodiments, specific types of cancers or malignant tumors, either primary or secondary, that can be treated using the methods, compositions, and formulations provided herein include, *e.g.*, leukemia, breast cancer, skin cancer, bone cancer, prostate cancer, liver cancer, lung cancer (*e.g.*, non-small-cell lung cancer and small-cell lung cancer), brain cancer, cancer of the larynx, gall bladder, pancreas, rectum, parathyroid, thyroid, adrenal, neural tissue, head and neck, colon, stomach, bronchi, kidneys, basal cell carcinoma, squamous cell carcinoma of both ulcerating and papillary type, metastatic skin carcinoma, osteo sarcoma, Ewing's sarcoma, veticulum cell sarcoma, myeloma, giant cell tumor, gallstones, islet cell tumor, primary brain tumor, acute and chronic lymphocytic and granulocytic tumors, hairy-cell tumor, adenoma, hyperplasia, medullary carcinoma, pheochromocytoma, mucosal neuronmas, intestinal ganglioneuromas, hyperplastic corneal nerve tumor, marfanoid habitus tumor, Wilm's tumor, seminoma, ovarian tumor, leiomyoma tumor, cervical dysplasia and in situ carcinoma, neuroblastoma, retinoblastoma, medulloblastoma, soft tissue sarcoma, malignant carcinoid, topical skin lesion, mycosis

fungoides, rhabdomyosarcoma, Kaposi's sarcoma, osteogenic and other sarcoma, malignant hypercalcemia, renal cell tumor, polycythermia vera, adenocarcinoma, glioblastoma multiforma, leukemias, lymphomas, malignant melanomas, epidermoid carcinomas, and other carcinomas and sarcomas.

[00198] Particular embodiments herein provide using the methods, compositions, and formulations provided herein to treat abnormal cell proliferation due to, *e.g.*, insults to body tissue during surgery for a variety of surgical procedures, including, *e.g.*, joint surgery, bowel surgery, and cheloid scarring. Proliferative responses associated with organ transplantation that may be treated using the methods, compositions, and formulations provided herein include those proliferative responses contributing to potential organ rejections or associated complications. Specifically, these proliferative responses may occur during transplantation of the heart, lung (*e.g.*, non-small-cell lung cancer and small-cell lung cancer), liver, kidney, and other body organs or organ systems.

[00199] In certain embodiments, the amount of the cytidine analog in the formulations provided herein, the methods of administration thereof, or the methods of treatment as set forth herein, is a specific dosage amount as provided herein. In certain embodiments, oral azacitidine dosages, methods of administration thereof, or methods of treatment of at least one condition, including but not limited to MDS and AML, may range, *e.g.*, between about 50 mg/m^2/day and about 2,000 mg/m^2/day, between about 100 mg/m^2/day and about 1,000 mg/m^2/day, between about 100 mg/m^2/day and about 500 mg/m^2/day, or between about 120 mg/m^2/day and about 250 mg/m^2/day. In certain embodiments, particular dosages are, *e.g.*, about 120 mg/m^2/day, about 140 mg/m^2/day, about 150 mg/m^2/day, about 180 mg/m^2/day, about 200 mg/m^2/day, about 220 mg/m^2/day, about 240 mg/m^2/day, about 250 mg/m^2/day, about 260 mg/m^2/day, about 280 mg/m^2/day, about 300 mg/ m^2/day, about 320 mg/m^2/day, about 350 mg/m^2/day, about 380 mg/m^2/day, about 400 mg/m^2/day, about 450 mg/m^2/day, or about 500 mg/m^2/day.

[00200] In certain embodiments, appropriate biomarkers may be used to determine or predict the effect of the pharmaceutical compositions comprising cytidine analogs on the disease state and to provide guidance to the dosing schedule. For example, particular embodiments herein provide a method of determining whether a patient diagnosed with MDS has an increased probability of obtaining a greater benefit from treatment with a pharmaceutical composition comprising a cytidine analog by assessing the patient's nucleic acid methylation status. In particular embodiments, the cytidine analog is azacitidine. In

particular embodiments, the nucleic acid is DNA or RNA. In particular embodiments, the greater benefit is an overall survival benefit. In particular embodiments, the methylation status is examined in one or more genes, *e.g.*, genes associated with MDS or AML. Specific embodiments involve methods for determining whether baseline DNA methylation levels influence overall survival in patients with MDS (*e.g.*, higher risk MDS) treated with azacitidine. Specific embodiments provide methods for determining whether gene promoter methylation levels influence overall survival in patients with MDS (*e.g.*, higher risk MDS).

[00201] For example, specific embodiments herein provide methods for evaluating the influence of gene methylation on prolonged survival in patients with MDS (*e.g.*, higher risk MDS). In particular embodiments, such evaluation is used to predict overall survival in patients with MDS (*e.g.*, higher risk MDS), *e.g.*, upon treatment with a pharmaceutical composition comprising a cytidine analog, as provided herein. In particular embodiments, such evaluation is used for therapeutic decision-making. In specific embodiments, such therapeutic decision-making includes planning or adjusting a patient's treatment, *e.g.*, the dosing regimen, amount, and/or duration of administration of the cytidine analogue.

[00202] Certain embodiments provide methods of identifying individual patients diagnosed with MDS having an increased probability of obtaining an overall survival benefit from cytidine analog treatment, using analysis of methylation levels, *e.g.*, in particular genes. In specific embodiments, lower levels of nucleic acid methylation are associated with an increased probability of obtaining improved overall survival following azacitidine treatment. In particular embodiments, the increased probability of obtaining improved overall survival following treatment is at least a 5% greater probability, at least a 10% greater probability, at least a 20% greater probability, at least a 30% greater probability, at least a 40% greater probability, at least a 50% greater probability, at least a 60% greater probability, at least a 70% greater probability, at least an 80% greater probability, at least a 90% greater probability, at least at least a 100% greater probability, at least a 125% greater probability, at least a 150% greater probability, at least a 175% greater probability, at least a 200% greater probability, at least a 250% greater probability, at least a 300% greater probability, at least a 400% greater probability, or at least a 500% greater probability of obtaining improved overall survival following treatment, *e.g.*, using a pharmaceutical composition comprising a cytidine analog as provided herein. In particular embodiments, the greater probability of obtaining improved overall survival following treatment is a greater probability as compared to the average probability of a particular comparison population of patients diagnosed with MDS.

In specific embodiments, the comparison population is a group of patients classified with a particular myelodysplastic subtype, as described herein. In one embodiment, the comparison population consists of patients having higher risk MDS. In particular embodiments, the comparison population consists of a particular IPSS cytogenetic subgroup.

[00203] In particular embodiments, nucleic acid (*e.g.*, DNA or RNA) hypermethylation status may be determined by any method known in the art. In certain embodiments, DNA hypermethylation status may be determined using the bone marrow aspirates of patients diagnosed with MDS, *e.g.*, by using quantitative real-time methylation specific PCR ("qMSP"). In certain embodiments, the methylation analysis may involve bisulfite conversion of genomic DNA. For example, in certain embodiments, bisulfite treatment of DNA is used to convert non-methylated CpG sites to UpG, leaving methylated CpG sites intact. *See, e.g.*, Frommer, M., *et al.*, *Proc. Nat'l Acad. Sci. USA* 1992, 89:1827-31. Commercially available kits may be used for such bisulfite treatment. In certain embodiments, to facilitate methylation PCR, primers are designed as known in the art, *e.g.*, outer primers which amplify DNA regardless of methylation status, and nested primers which bind to methylated or non-methylated sequences within the region amplified by the first PCR. *See, e.g.*, Li *et al.*, *Bioinformatics* 2002, 18:1427-31. In certain embodiments, probes are designed, *e.g.*, probes which bind to the bisulfite-treated DNA regardless of methylation status. In certain embodiments, CpG methylation is detected, *e.g.*, following PCR amplification of bisulfite-treated DNA using outer primers. In certain embodiments, amplified product from the initial PCR reaction serves as a template for the nested PCR reaction using methylation-specific primers or non-methylation-specific primers. In certain embodiments, a standard curve is established to determine the percentage of methylated molecules in a particular sample. Methods for detecting nucleic acid methylation (*e.g.*, RNA or DNA methylation) are known in art. *See, e.g.*, Laird, P.W., *Nature Rev. Cancer* 2003, 3:253-66; Belinsky, S.A., *Nature Rev. Cancer* 2004, 4:1-11.

[00204] In certain embodiments, statistical analyses are performed to assess the influence of particular methylation levels with the potential benefit of treatment with a particular pharmaceutical composition comprising a cytidine analog. In certain embodiments, the influence of methylation on overall survival is assessed, *e.g.*, using Cox proportional hazards models and Kaplan-Meier (KM) methodology.

[00205] In certain embodiments, any gene associated with MDS and/or AML may be examined for its methylation status in a patient. Particular genes include, but are not limited

to, *CKDN2B (p15)*, *SOCS1*, *CDH1 (E-cadherin)*, *TP73*, and *CTNNA1 (alpha-catenin)*. Particular genes associated with MDS and/or AML, which would be suitable for use in the methods disclosed here, are known in the art.

1. Methods Comprising Co-Administering One or More Additional Therapeutic Agents with the Oral Formulations Disclosed Herein

[00206] Certain embodiments herein provide methods of treating diseases or disorders disclosed herein (*e.g.*, diseases or disorders involving abnormal cell proliferation), wherein the methods comprise co-administering an oral formulation disclosed herein (such as, for example, an oral formulation comprising 5-azacytidine) with one or more additional therapeutic agents (such as, for example, a cancer therapeutic agent) to yield a synergistic therapeutic effect. Particular co-administered therapeutic agents useful in the methods disclosed herein are disclosed throughout the specification. In particular embodiments, the additional therapeutic agent is co-administered in an amount that is a therapeutically effective amount. In particular embodiments, the additional therapeutic agent is co-administered in a separate dosage form from the cytidine analog dosage form with which it is co-administered. In particular embodiments, the additional therapeutic agent is co-administered in a dosage form (*e.g.*, a single unit dosage form) together with the cytidine analog with which it is co-administered. In such cases, the cytidine analog (*e.g.*, azacitidine) and the additional therapeutic agent may be co-formulated together in the same dosage form using methods of co-formulating active pharmaceutical ingredients, including methods disclosed herein and methods known in the art.

[00207] Incorporation By Reference: All disclosures (*e.g.*, patents, publications, and web pages) referenced throughout this specification are incorporated by reference in their entireties. In addition, the following disclosures are also incorporated by reference herein in their entireties: (1) 2008 ASCO poster abstract by B. S. Skikne, M. R. Ward, A. Nasser, L. Aukerman, G. Garcia-Manero; and (2) G. Garcia-Manero, M. L. Stoltz, M. R. Ward, H. Kantarjian, and S. Sharma, Leukemia, 2008, 22, 1680-84.

VII. EXAMPLES

A. Example 1

[00208] 5-Azacytidine tablets were manufactured using direct tablet compression followed by optional seal film-coating and/or enteric film-coating, as described below. **Table 3** lists the excipients used in each of the tablet formulations. **Table 4** describes the formula composition of the tablets using weights. **Table 5** describes the formula composition of the tablets using percentages.

[00209] Formulation 1 was manufactured without the seal-coating step, which may have resulted in an enteric coat that contained a "leaky" enteric coating. Talc was only used in the enteric coating suspension for Formulation 1.

[00210] Except for Formulation 1, a common blend with 20% drug load of 5-azacytidine was used to manufacture all tablets. Vitamin E TPGS (d-alpha-tocopheryl polyethylene glycol 1000 succinate) was added to certain of the formulations to enhance absorption of 5-azacytidine. Vitamin E TPGS was not used in Formulation 6.

[00211] Tablets were manufactured using the process described Figure 1, except for Formulation 1 (which did not undergo the seal-coating step). Formulations 3 and 6 did not undergo the enteric film-coating step, and Formulation 6 did not contain Vitamin E TPGS. The process is generally described as follows:

[00212] Mannitol, silicified microcrystalline cellulose, crospovidone, magnesium stearate and azacitidine were individually screened to ensure de-aggregation of any agglomerates. Vitamin E TPGS was melted in a stainless steel vessel to which was then added a portion of the silicified microcrystalline cellulose (not done in Formulation 6). The Vitamin E TPGS - silicified microcrystalline cellulose mixture was allowed to cool and then screened. Azacitidine, Vitamin E TPGS - silicified microcrystalline cellulose mix, remaining silicified microcrystalline cellulose, mannitol and crospovidone were mixed in a V-blender. Magnesium stearate was added to the V-blender followed by additional mixing. The resulting blend was compressed into tablets using standard concave tooling.

[00213] Hydroxypropyl cellulose was dispersed into ethanol. The hydroxypropyl cellulose preparation was used to spray coat the tablet cores to prepare seal coated tablets.

[00214] EUDRAGIT and triethyl citrate were dispersed into an isopropanol - acetone mixed solvent system. EUDRAGIT - triethyl citrate preparation was used to spray coat the seal coated tablet.

TABLE 3: Components of Azacitidine Tablets

Component	Function	Quality Standard
Azacitidine	API	In-House
Mannitol	Bulking Agent	USP
Silicified Microcrystalline Cellulose	Binding Agent	NF
d-alpha-tocopheryl polyethylene glycol 1000 succinate (Vitamin E TPGS)	Permeation Enhancer	NF
Polyvinyl Polypyrrolidone (Crospovidone)	Disintegrant	NF
Magnesium Stearate	Lubricant	NF
Hydroxypropyl Cellulose	Seal Film Coat	NF
Ethanol [a]	Coating Solvent	USP
Methacrylic Acid Copolymer (Eudragit S100, Eudragit LIDO-55 or Eudragit L100)	Enteric Film Coat	NF
Triethyl Citrate	Plasticizer	NF
Talc	Anti-Caking	USP
Isopropanol [a]	Coating Solvent	USP
Acetone	Coating Solvent	NF

[a] Removed during processing (used as solvent for film-coating polymers).

TABLE 4: Formula Composition of Azacitidine Tablets (Weight)

Component	Quantity per Unit Tablet (mg)					
	Formulation #1 Leaky coating (pH > 7.0)	**Formulation #2** Enteric-coated (pH > 7.0)	**Formulation #3** Immediate Release w/ vitamin E	**Formulation #4** Enteric-coated (pH > 5.0)	**Formulation #5** Enteric-coated (pH > 5.5)	**Formulation #6** Immediate Release w/o vitamin E
Azacitidine[a]	20.0	20.0	60.0	60.0	60.0	60.0
Mannitol, USP	59.7	43.2	129.6	129.6	129.6	135.6
Silicified Microcrystalline Cellulose, NF	13.9	30.0	90.0	90.0	90.0	90.0
Crospovidone, NF	2.8	3.0	9.0	9.0	9.0	9.0
Magnesium Stearate, NF	1.6	1.8	5.4	5.4	5.4	5.4
Vitamin E TPGS, NF	2.0	2.0	6.0	6.0	6.0	0
Core Tablet Total	100.0	100.0	300.0	300.0	300.0	300.0
Hydroxypropyl Cellulose, NF	N/A	4.0	12.0	12.0	12.0	12.0
Ethanol[b]	N/A	–	–	–	–	–
Seal-Coated Tablet Total	N/A	104.0	312.0	312.0	312.0	312.0
Eudragit S-100	3.7 – 5.9	7.0 –8.0	N/A	N/A	N/A	N/A
Eudragit L 100-55	N/A	N/A	N/A	21.8 – 25.0	N/A	N/A
Eudragit L 100	N/A	N/A	N/A	N/A	28.1 – 31.2	N/A
Triethyl Citrate	0.3 – 0.5	1.0 – 2.0	N/A	3.0 – 6.0	3.0 – 6.0	N/A
Talc	1.0 – 1.6	N/A	N/A	N/A	N/A	N/A
Isopropanol[b]	–	–	N/A	–	–	N/A
Acetone[b]	–	–	N/A	–	–	N/A
Total Theoretical Weight	106.5	113.0	312.0	335.4	341.64	312.0

[a] Assuming 100% purity.
[b] Removed during processing.

TABLE 5: Formula Composition of Azacitidine Tablets (Percent)

Component	Quantity per Unit Tablet (mg)					
	Formulation #1 Leaky coating (pH > 7.0)	**Formulation #2** Enteric-coated (pH > 7.0)	**Formulation #3** Immediate Release w/ vitamin E	**Formulation #4** Enteric coated (pH > 5.0)	**Formulation #5** Enteric coated (pH > 5.5)	**Formulation #6** Immediate Release w/o vitamin E
Azacitidine [a]	20.0	20.0	20.0	20.0	20.0	20.0
Mannitol, USP	59.7	43.2	43.2	43.2	43.2	45.2
Silicified Microcrystalline Cellulose, NF	13.9	30.0	30.0	30.0	30.0	30.0
Crospovidone, NF	2.8	3.0	3.0	3.0	3.0	3.0
Magnesium Stearate, NF	1.6	1.8	1.8	1.8	1.8	1.8
Vitamin E TPGS, NF	2.0	2.0	2.0	2.0	2.0	0.0
Core Tablet Total	100.0	100.0	100.0	100.0	100.0	100.0
Hydroxypropyl Cellulose, NF	N/A	4.0	4.0	4.0	4.0	4.0
Ethanol [b]	N/A	–	–	–	–	–
Seal-Coated Tablet Total		104.0	104.0	104.0	104.0	104.0
Eudragit S-100	3.7 – 5.9	7.0 –8.0	N/A	N/A	N/A	N/A
Eudragit L 100-55	N/A	N/A	N/A	7.0 –8.0	N/A	N/A
Eudragit L 100	N/A	N/A	N/A	N/A	9.0 –10.0	N/A
Triethyl Citrate	0.3 – 0.5	1.0 – 2.0	N/A	1.0 – 2.0	1.0 – 2.0	N/A
Talc	1.0 – 1.6	N/A	N/A	N/A	N/A	N/A
Isopropanol [b]	–	–	N/A	–	–	–
Acetone [b]	–	–	N/A	–		–

[a] Assuming 100% purity.
[b] Removed during processing

B. **Example 2**

[00215] Studies were performed to evaluate the effect of aqueous film coating on hydrolytic degradation of azacitidine. Azacitidine tablets were film-coated using aqueous-based solvents without affecting levels of degradation. As demonstrated in **Table 6**, significant levels of azacitidine degradation products were not observed after aqueous film coating.

TABLE 6. Effect of Aqueous Film Coating on Azacitidine

Test	Uncoated Core Tablet	Coated Tablet
Assay (% Label Claim)	Ave=103.1	Ave=99.6
Related Substances (% Area)		
N-Formylguanylribosylurea	0.2	0.1
Guanylribosylurea	0.7	0.7
Unspecified	ND	ND
Total	0.9	0.8
Moisture Content (% w/w)	NMT 2.5	2.2

ND = Not detected; NMT = No more than

C. **Example 3**

[00216] As described in Example 1, the following six formulations, described in **Table 7** and elsewhere in the present specification, were prepared and used in clinical studies as described in the Examples below:

TABLE 7. Formulations of Azacitidine used in clinical studies

Formulation Number	Azacitidine in Formulation	Description
#1	20 mg	"Leaky" enteric-coated tablet
#2	20 mg	Enteric-coated tablet, core sealed
#3	60 mg	Seal-coated, immediate release tablet with vitamin E
#4	60 mg	Enteric film-coated tablet, target dissolution at pH > 5.5
#5	60 mg	Enteric film-coated tablet, target dissolution at pH > 6.0
#6	60 mg	Seal-coated, immediate release tablet without vitamin E

D. **Example 4**

[00217] In a multiple dose escalation study (MTD study; CL005), patients with MDS or AML were selected (Selection criteria: ECOG PS 0-2, adequate organ function, age >18 years). The patients were dosed with multiple 28-day cycles of azacitidine. The study had a 3+3 design. During Cycle 1, all patients were dosed subcutaneously with azacitidine at 75 mg/m^2 x 7 days. During subsequent cycles (dosing on Day 1-7 for each cycle), the patients were dosed orally with azacitidine at doses listed in **Table 8**. PK data were collected during Cycles 1 and 2 on Day 1 and 7, and during Cycles 4, 5, and 7, on Day 7. PD data were collected during each cycle, and hematological responses and/or improvement rates were assessed for each treatment cycle to determine biologically active dose (BAD). To date, seven cohorts of patients (3 subjects/cohort) have been studied and none of the patients have shown dose limited toxicity (DLT). The oral dose and formulation used for each cohort are listed in **Table 8**.

TABLE 8. Oral Azacitidine Doses and Formulations

Cohort # Dosage	Oral Formulation	Subject Demographics (Patient No. – gender, age, dx)	# Subjects Treated/Evaluable for DLT	# Subjects with DLT
Cohort 1 120 mg	Formulation #2 (20 mg tablets)	02001 – M, 78, MDS 02002 – M, 66, MDS RAEB-2 04001 – M, 56, MDS RAEB-1	3 / 3	0
Cohort 2 120 mg	Formulation #1 (20 mg tablets)	02003 – M, 73, AML 02004 – M, 61, MDS 04002 – M, 73, MDS RAEB-1 02005 – M, 66, MDS RAEB-1	4 / 3	0
Cohort 3 180 mg	Formulation #1 (20 mg tablets)	04004 – F, 70, AML 02006 – M, 61, AML 03001 – F, 70, MDS RAEB-2	3 / 3	0
Cohort 4 240 mg	Formulation #3 (60 mg tablets)	02007 – M, 76, CMML 02008 – M, 80, MDS RAEB-1 02009 – M, 83, MDS RAEB-2	3 / 3	0
Cohort 5 300 mg	Formulation #3 (60 mg tablets)	04005 – M, 68, MDS RCMD 02011 – M, 92, MDS RAEB-1 02012 – M, 62, MDS RCMD	3 / 3	0
Cohort 6 360 mg	Formulation #3 (60 mg tablets)	02013 – F, 66, MDS RAEB-1 03002 – M, 65, MDS RAEB-1 01001 – F, 63, MDS RCMD	3 / 3	0
Cohort 5 480 mg	Formulation #3 (60 mg tablets)	01002 – M, 70, MDS RARS 01003 – F, 75, MDS RCMD	2 / 0*	0

* Cycle 2 ongoing

[00218] PK profiles for Cycle 1, following 75 mg/m^2 SC dose of azacitidine, are presented in Figure 2. Pharmacokinetic parameters calculated from azacitidine plasma concentrations following SC doses at 75 mg/m^2 are presented in **Table 9**.

TABLE 9. PK parameters from Cycle 1, following SC doses at 75 mg/m^2

		AUC(0-t) (ng*hr/mL)	AUC(0-inf) (ng*hr/mL)	Cmax (ng/mL)	Tmax (hr)	Lambda_z (1/hr)	t1/2 (hr)	Cloral (L/hr)	Vdoral (L)
Day 1	Mean (n=18)	1135	1170	741	0.49	0.58	1.53	143	318
	SD	514	533	293	0.27	0.29	0.80	53	223
	Minimum	505	538	224	0.23	0.22	0.61	45	90
	Median	991	1030	674	0.50	0.56	1.24	156	265
	Maximum	2821	2950	1310	1.08	1.14	3.15	253	788
	CV%	45	46	39	54	49	52	37	70
Day 7	Mean (n=18)	1135	1210	697	0.51	0.62	1.73	133	368
	SD	477	463	252	0.17	0.39	1.28	43	376
	Minimum	510	686	254	0.25	0.16	0.47	48	98
	Median	1020	1116	716	0.50	0.55	1.26	148	162
	Maximum	2718	2783	1050	1.00	1.49	4.30	223	1383
	CV%	42	38	36	34	62	74	33	102

[00219] Plasma PK profiles following SC (75 mg/m^2) and various PO doses are compared and presented in Figure 3. An increase in oral dose did not result in dose-proportional increase in exposure of azacitidine.

[00220] Methylation PD data in cycles 1 and 2, from blood (PBL) and bone marrow (BM) samples, were obtained. The PD data collected from individual patients from Cohort 4 (Formulation #3, oral dose 240 mg) are presented in Figure 4 and Figure 5.

[00221] Subject number 02004 of cohort 2 (61-year-old male with MDS, MDACC) was treated with a SC cycle of azacitidine, followed by initial oral doses of 120 mg azacitidine (Formulation #1). The patient received oral doses of 120 mg x 7d azacitidine as in Formulation #1 during Cycles 2-6, followed by oral doses of 180 mg x 7d azacitidine during Cycles 7-12. In this patient, following a 75 mg/m^2 SC dose of azacitidine, the AUC value was 1000 ng*hr/mL. Following a 180 mg oral dose of azacitidine, the AUC value was 330 ng*hr/mL, approximately 33% of the exposure observed for the SC dose (oral bioavailability = 30%).

[00222] The PD response data from patient 02004 is presented in Figure 6. Platelets (K/uL), Hgb (g/dL), ANC (K/uL), and Relative BM Blast (%) are plotted vs. sampling dates

over the course of the study. The patient demonstrated a morphologic complete response (CR).

[00223] For patient 02004, Hgb (10.8 g/dL at ascreening, 11.1 g/dL at Day 1), Platelets (140 K/uL at both screening and Day 1), ANC (1.46 K/uL at screening and 1.12 K/uL at Day 1), and BM Blast (2%) values at baseline and Day 1 were above normal or close to normal. This patient had no transfusion (RBC or PLT) prior to enrollment into the study and to day required no transfusions (RBC or PLC) during the study. Per IWG 2006 criteria, the patient achieved complete response (CR) (from Days 45 – 74 satisfying all CR criteria for 28 consecutive days). The patient achieved morphologic complete response per the IWG AML criteria. However, with regard to the ANC condition for the IWG 2000 CR criteria, the patient did not meet the criteria for a complete response (3 days short of the duration requirement of 56 consecutive days).

[00224] For patient 02007, as shown in Figure 5, Grade 4 thrombocytopenia and neutropenia developed during the first cycle of treatment with subcutaneous azacitidine when given at 75 mg/m^2 for 7 days. The onset of the cytopenias occurred between days 14 and 21 at time points consistent with the existing safety profile of azacitidine when administered 75 mg/m^2 for 7 days as a SC injection. In contrast, the administration of oral azacitidine starting with cycle 2 did not result in grade 3 or 4 cytopenias yet still produced an increase in platelets above the baseline levels. This data supports, *e.g.*, the conclusion that certain oral dosage forms provided herein permit the delivery of azacitidine at lower doses over a more prolonged period of time, and that certain oral dosage forms provided herein alter the safety profile of the cytidine analog.

[00225] Assessment of IWG criteria for certain patients in the MTD study is presented below in **Table 10**. The data demonstrate, *inter alia*, patient improvement following administration of azacitidine formulated for release substantially in the stomach.

Table 10. MTD Study; Assessment of IWG Criteria

Patient No.	IWG Assessment
02004	• Fairly healthy at baseline: hgb (11.1 g/dL Cycle 1, Day 1); PLT (140 K/μL Cycle 1, Day 1); ANC (1.12 K/μL at Cycle 1, Day 1); BM blasts (2%) values at baseline above normal or close to normal • CR per IWG 2006 (Days 45-98) • Morphologic CR per the IWG AML criteria (Diagnosis is MDS)
02007	• HI-P major improvement per IWG 2000 (Days 35-202) • Morphologic CR per the revised IWG AML criteria on Days 43-188 and on some other days (ANC=1.89 K/μL, but normal at BL=2.99 and 1.68; PLT=314 K/μL; BM=2, but normal at BL=3) (Diagnosis is CMML)
02008	• HI-P major improvement per IWG 2000 (Days 34-110)
02009	• Marrow CR (Days 7-111+) per IWG 2006
02011	• Marrow CR (Days 7-177+) per IWG 2006 • Morphologic CR per the revised IWG AML criteria on Day 21 (ANC=1.18 K/μL; PLT=119 K/μL, but normal at BL=162 & 194; BM=3) (Diagnosis is MDS)

[00226] Immediate release oral formulations comprising azacitidine demonstrated bioavailability in patients. Observations thus far suggest positive clinical activity in patients treated with oral azacitidine formulations. No safety issues have thus far been observed with the doses and schedules described above.

E. Example 5

[00227] An oral azacitidine clinical study, referred to as the Rapid Aza Clinical Evaluation (RACE) study (CL008), was performed; a summary of the study design is depicted in Figure 7. Several oral formulations were evaluated in this study. A "3 + 7" cohort of patients was enrolled in the study, *i.e.*, three patients were initially tested per formulation, and the cohorts could increase in size up to ten patients. Cohorts were enrolled in parallel. PK data was collected periodically, as indicated in **Table 11**.

TABLE 11. RACE Study – PK Study Design; PK Cycle 1, Days 1, 3, 5, 15, 17 & 19, and Cycle 2, Day 7

Treatment Day	Dose
PK Phase (Cycle 1)	
Day 1	75 mg/m^2 SC
Day 3+1*	180 mg Oral
Day 5±1*	360 mg Oral
Day 15±1*	75 mg/m^2 SC
Day 17±1*	Oral dose calculated to achieve approximately 80% exposure relative to the 75 mg/m^2 SC dose up to a maximum dose of 1,200 mg.
Day 19±1*	Oral dose calculated to achieve approximately 120% exposure relative to the 75 mg/m^2 SC dose up to a maximum dose of 1,200 mg.
Treatment Phase (Cycles 2 – 7)	
Days 1-7	Oral dose calculated to achieve approximately 100% exposure relative to the 75 mg/m^2 SC dose up to a maximum dose of 1,200 mg.

* Dose administered ± 1 day, as long at least 48 hours between doses

[00228] Results for Formulation #4: The plasma PK profile for one subject who received Formulation 4 (*i.e.*, enteric film-coated tablets for release in upper gastrointestinal region) is depicted in Figure 8. Values for AUC (0-t) (ng*hr/mL) were as follows: SC administration of 75 mg/m^2 (124 mg) = 2390 (day 1) and 2440 (day 15); Oral administration of 180 mg = 234; Oral administration of 360 mg = 197; and Oral administration of 1200 mg = 66.5 (day 17) and 297 (day 19). Tmax for oral administration was reached between 2.5 hr and 3.0 hr. A linear increase of exposure (AUC0-inf) was not observed following 180, 360, and 1200 mg oral doses. Relative oral bioavailability ranged between 0.8 to 6.7%.

[00229] Results for Formulation #6: The plasma PK profile for one subject who received Formulation 6 (*i.e.*, seal-coated immediate release tablets without vitamin E) is depicted in Figure 9. Values for AUC(0-∞) (ng*hr/mL) were as follows: SC administration of 75 mg/m^2 (120 mg) = 1720 (day 1) and 1640 (day 15); Oral administration of 180 mg = 231; Oral administration of 360 mg = 280; and Oral administration of 1200 mg = 543 (day 17) and 467 (day 19). Tmax for oral administration was reached between 0.5 hr and 1.0 hr. A linear increase of exposure (AUC 0-∞) was observed following 180, 360, and 1200 mg oral doses, although the increase was not proportional with dose. Following the 1200 mg oral doses, AUC was approximately 30% of AUC following SC dosing (*i.e.*, about 500 and about 1,700, respectively).

[00230] Data from this study indicated that azacitidine was absorbed following oral administration of immediate release formulations comprising azacitidine. As compared to SC administration of azacitidine, the immediate release azacitidine formulations provided a superior percent exposure (*e.g.*, about 30%) than enteric-coated azacitidine formulations. Data supports single or multiple daily dosing of oral azacitidine.

F. Example 6

[00231] Based on data from clinical studies involving different azacitidine formulations and dosage amounts, plots were prepared comparing different formulations with respect to, *e.g.*, their resulting PK profiles, AUC values, Cmax values, relative oral bioavailability values, and exposure values. Oral formulations involved in the comparisons include Formulation #3 ("F3"); Formulation #4 ("F4"); and Formulation #6 ("F6"); these oral formulations are described elsewhere herein (*e.g.*, in Examples 1 and 3).

[00232] Comparisons of Formulation Nos. 3, 4, and 6

[00233] Figure 10 compares PK profiles (using a linear scale) following administration of azacitidine via SC (75 mg/m^2; n=18) and oral administration. For Formulation #3, a total of 360 mg azacitidine was orally administered (n=6); for Formulation #4, a total of 360 mg azacitidine was orally administered (n=3); for Formulation #6, a total of 360 mg azacitidine was orally administered (n=5). The plot illustrates immediate release characteristics of Formulations #3 and #6, as compared to Formulation #4, which was enteric coated. Figure 11 provides the same data, plotted on a semi-log scale.

[00234] Patients were dosed with azacitidine SC (75 mg/m^2) and orally with Formulations #3, #4, or #6 ("F3"; "F4"; and "F6"; described elsewhere herein) with a total of 180 mg, 240 mg, 300 mg, 360 mg, 540 mg, 600 mg, 720 mg, 900 mg, 1080 mg, or 1200 mg azacitidine administered per patient. Results showed that azacitidine is absorbed following oral administration. As described below, particular values were measured and compared, including AUC values, Cmax values, relative oral bioavailability values, and exposure values (oral) compared to SC.

[00235] Figure 12 displays AUC values (ng*hr/mL; mean ± SD) following azacitidine administration. Figure 13 displays Cmax values (ng/mL; mean ± SD) following azacitidine administration. For Formulation #4 (enteric coated), over the dose range of 180 mg to 1200 mg, an increase in dose did not translate into an increase in exposure, and absorption was poor. For Formulation #3 (immediate release tablets with vitamin E), over the dose range of 180 mg to 1200 mg, an increase in dose translated into an increase in exposure. For

Formulation #6 (immediate release tablets without vitamin E), over the dose range of 180 mg to 1200 mg, an increase in dose translated into an increase in exposure. Tmax for immediate-release Formulations #3 and #6 were similar: For Formulation #3, median Tmax was 1.1 hr (range 0.5, 2.5 hr); For Formulation #6, median Tmax was 1.0 hr (range 0.5, 3.0 hr).

[00236] Figure 14 displays relative oral bioavailability (%; mean ± SD) following oral dosing with Formulations #3, #4, and #6, at various azacitidine dosage levels. At dosage levels less than or equal to 360 mg azacitidine, Formulation #4 (enteric coated) had a mean relative oral bioavailability of less than 4%. At dosage levels less than or equal to 360 mg azacitidine, Formulation #3 (immediate release with vitamin E) had a mean relative oral bioavailability ranging from 11% to 21%. At dosage levels less than or equal to 360 mg azacitidine, Formulation #6 (immediate release without vitamin E) had a mean relative oral bioavailability ranging from 11% to 14%.

[00237] Figure 15 displays exposure (% as compared to SC; mean ± SD) following oral dosing with Formulations #3, #4, and #6, at various azacitidine dosage levels. At dosage levels less than or equal to 360 mg azacitidine, Formulation #4 (enteric coated) had a mean exposure of less than 8%. At dosage levels less than or equal to 360 mg azacitidine, Formulation #3 (immediate release with vitamin E) had a mean exposure ranging from 18% to 37%. At dosage levels less than or equal to 360 mg azacitidine, Formulation #6 (immediate release without vitamin E) had a mean exposure ranging from 20% to 31%. As compared to enteric-coated Formulation #4, the immediate-release Formulations #3 and #6 provided superior exposure compared to SC (about 30% at total dosage amount of 360 mg).

[00238] Comparisons of Formulation Nos. 3 and 6

[00239] Figure 16 displays a linear scale profiles of azacitidine plasma concentration (ng/ml) versus time (hr) for Formulation #3 and #6 at a dosage level of 180 mg (n=6). Figure 17 displays linear scale profiles of azacitidine plasma concentration (ng/ml) versus time (hr) for Formulation #3 and #6 at a dosage level of 360 mg (n=6).

[00240] Figure 18 displays a plot of individual ("ind") and mean azacitidine AUC(0-inf) (ng*hr/ml) versus azacitidine dose (mg) for Formulation #3 and #6, with linear regression analysis. Linear regression equations for F3 and F6 are also indicated on the plot. Using those equations, for a selected dose, the expected AUC(0-inf) (ng*hr/ml) were calculated. Calculated values are provided in **Table 12**.

TABLE 12. Expected AUC(0-inf) Calculated for Formulation #3 and #6

	AUC(0-inf) (ng*hr/ml)	
Azacitidine Dose (mg)	**Formulation #6**	**Formulation #3**
240	263	338
360	296	363
480	328	388
600	361	413
720	393	438
1200	523	538
F6 linear regression equation: $y = 0.2706 x + 198.19$		
F3 linear regression equation: $y = 0.2079 x + 288.07$		

[00241] Figure 19 displays a comparison of azacitidine percent relative oral bioavailability (mean ± SD) versus azacitidine dose (mg), following dosing with Formulation #3 or #6, for azacitidine oral dosage amounts including 180 mg, 240 mg, 300 mg, 360 mg, 480 mg, 600 mg, 720 mg, 900 mg, 1020 mg, 1080 mg, 1140 mg, and 1200 mg. At doses greater than or equal to 1020 mg, the mean relative oral bioavailability for Formulation #6 ranged from 9% to 14%, and the mean relative oral bioavailability for Formulation #3 ranged from 10% to 21%.

[00242] Figure 20 displays a comparison of azacitidine percent oral exposure as compared to SC azacitidine dosing (mean ± SD) versus azacitidine dose (mg), following oral administration of Formation #3 or #6. Azacitidine oral dosage amounts included 180 mg, 240 mg, 300 mg, 360 mg, 480 mg, 600 mg, 720 mg, 900 mg, 1020 mg, 1080 mg, 1140 mg, and 1200 mg. At doses with n>1, the mean exposures of Formulation #6 and #3, as compared to SC, were similar.

G. Example 7

[00243] DNA methylation was employed as a biomarker to monitor responses in patients treated with azacitidine in the clinical studies described herein. Analysis was performed with an Infinium Assay (commercially available from Illumina, Inc., San Diego, California). The Infinium Assay combined with BeadChips allows large-scale interrogation of variations in the human genome. For example, the Infinium HumanMethylation27 BeadChip enables interrogation of 27,578 CpG loci, covering over 14,000 genes. The DNA Methylation Assay

protocol included the following steps: (1) bisulfite conversion; (2) DNA amplification; (3) DNA fragmentation; (4) DNA precipitation; (5) DNA hybridization to BeadChip; (6) extension and staining on BeadChip; and (7) imaging of BeadChip.

[00244] The assay for methylation was used to detect methylation status at individual CpG loci by typing bisulfite-converted DNA. Methylation protected C from conversion, whereas unmethylated C was converted to T. A pair of bead-bound probes was used to detect the presence of T or C by hybridization followed by single-base extension with a labeled nucleotide. Up to twelve samples were profiled in parallel. Blood and bone marrow samples were collected and DNA methylation was analyzed in parallel.

H. Example 8

[00245] A study is performed to examine whether baseline DNA and/or RNA methylation levels influence overall survival (OS) as well as the interaction between gene promotor methylation levels and treatment (*e.g.*, azacitidine or conventional care regimens ("CCR")). Methylation is determined for 5 genes previously evaluated in MDS or AML: *CDKN2B (p15)*, *SOCS1*, *CDH1 (E-cadherin)*, *TP73*, and *CTNNA1 (alpha-catenin)*, in pre-treatment bone marrow aspirates of patients enrolled in a clinical study using quantitative real-time methylation specific PCR (qMSP). The influence of methylation on OS is assessed using Cox proportional hazards models and Kaplan-Meier (KM) methodology.

[00246] The number of patients (*e.g.*, for azacitidine and CCR) having nucleic acid sufficient for analysis of these 5 genes is determined. Methylation is detected in a specific percentage of patients for *CDKN2B, SOCS1*, *CDH1*, *TP73*, and *CTNNA1*. Differences in methylation levels between the treatment arms are determined. The OS benefit for cytidine analog (*e.g.*, azacitidine) treatment is determined for patients who are positive and negative for methylation at these 5 genes. It is determined whether the presence of methylation is associated with improvement in OS in the CCR group (prognostic indicator of good outcome). The existence and magnitude of any effect is compared to the cytidine analog group, which may suggest an interaction between DNA and/or RNA methylation and treatment.

[00247] OS improvement is assessed with cytidine analog (*e.g.*, azacitidine) treatment in patients with methylation at any of these 5 genes, and HR of death for methylation is determined. The frequency of methylation of particular genes allows for examination of the influence of methylation level on OS and treatment effect. For example, for particular genes, lower levels of methylation may be associated with the longest OS and the greatest OS

benefit from cytidine analog treatment, compared with the absence of methylation. Influence of methylation level on OS may be assessed in each IPSS cytogenetic subgroup (good, intermediate, and poor). For example, the influence of methylation on OS may be strongest in the "poor" risk group, where risk of death is greatest.

[00248] Such data and analysis may indicate, *e.g.*, that patients with lower levels of methylation may derive greater benefit from treatment with pharmaceutical compositions comprising a cytidine analog (*e.g.*, azacitidine). Molecular biomarkers may be important in MDS, *e.g.*, as indicators of disease prognosis and predictors of response to epigenetic therapy.

I. Example 9

[00249] Clinical studies are conducted to assess the ability of an oral formulation comprising a cytidine analog, such as 5-azacytidine, to treat patients having lung cancer, *e.g.*, non-small-cell lung cancer (NSCLC). Such studies may include, *e.g.*, an assessment of the ability to stop or reverse the growth of particular NSCLC cell types in patients having NSCLC). In certain clinical studies, patients are tested for particular NSCLC cell types, *e.g.*, A549, H1975, H522, H23, H460, and H1299, prior to administration of the oral formulation. In certain clinical studies, patients with cell types known or believed to benefit preferentially from cytidine analog (*e.g.*, 5-azacytidine) administration may be enrolled. In certain clinical studies, patients having NSCLC are enrolled without analysis of particular NSCLC cell type. In certain clinical studies, patients having any type of NSCLC cells are candidates for treatment with an oral formulation provided herein.

[00250] In certain clinical studies, patients from any of the three main NSCLC groups may be enrolled, *i.e.*, (1) patients with tumors that are surgically resectable; (2) patients with either locally or regionally advanced lung cancer; or (3) patients with distant metastases at the time of diagnosis. In certain clinical studies, patients may be currently undergoing additional treatment for NSCLC, including, *e.g.*, surgery, chemotherapy, or radiation therapy.

[00251] In certain clinical studies, patients who are administered an oral formulation comprising a cytidine analog (*e.g.*, 5-azacytidine) may also be administered one or more additional therapeutic agents, examples of which are disclosed herein. The additional therapeutic agent(s) may be administered in the same oral formulation as the cytidine analog, or may be co-administered (*e.g.*, via PO, SC or IV administration) in combination with an oral formulation comprising the cytidine analog. The appropriate amount and dosing

schedule for an additional therapeutic agent is determined for a particular patient using methods known in the art.

[00252] An association between gene methylation and recurrence of NSCLC tumors is known in the art. *See, e.g.*, M.V. Brock *et al.*, *N. Engl. J. Med.*, 2008, 358(11):1118-28. Accordingly, in certain clinical studies provided herein, patients are screened prior to enrollment and/or monitored during the trial for DNA or RNA methylation levels, which indicate a potential response to treatment with an oral formulation comprising a cytidine analog (*e.g.*, 5-azacytidine). In certain clinical studies, patients with high levels of DNA methylation (*e.g.*, CpG island methylation) and/or an increased potential for transcriptional silencing of tumor-suppressor genes may be administered a cytidine analog (*e.g.*, 5-azacytidine) known or believed to prevent or reverse hypermethylation (*e.g.*, by reducing the activity of one or more DNA methyltransferase enzymes). In such studies, patients may also be co-administered one or more additional therapeutic agents known or believed to reduce epigenetic silencing, such as, *e.g.*, compounds that inihibit histone deacetylase enzymes (HDACs), which regulate the acetylation and deacetylation of histone residues that increase or decrease gene expression. *See, e.g.*, J.G. Herman & S.B. Baylin, *N. Engl. J. Med.*, 2003, 349:2042-54; P.A. Jones & S.B. Baylin, *Nature Rev. Gen.*, 2002, 3:415-28. Suitable HDAC inhibitors for co-administration in the clinical studies disclosed herein are known in the art and/or described herein (*e.g.*, entinostat or vorinostat).

[00253] The amount of cytidine analog (*e.g.*, 5-azacytidine) in the oral formulations administered during the clinical studies depends, *e.g.*, on the individual characteristics of the patient, including, *inter alia*, the stage and progression of the patient's NSCLC, the patient's age and weight, the patient's prior treatment regimens, and other variables, as known in the art. In certain clinical studies, potential starting doses may be, *e.g.*, about 60 mg, about 120 mg, about 180 mg, about 240 mg, about 300 mg, about 360 mg, about 420 mg, about 480 mg, about 540 mg, about 600 mg, about 660 mg, about 720 mg, about 780 mg, about 840 mg, about 900 mg, about 960 mg, about 1020 mg, or greater than about 1020 mg of the cytidine analog (*e.g.*, 5-azacytidine) daily for a specified time period, *e.g.*, about 1 week, about 1.5 weeks, about 2 weeks, about 2.5 weeks, about 3 weeks, about 3.5 weeks, about 1 month, about 1.5 months, about 2 months, or a longer time period. Other potential starting doses and time periods are disclosed herein. Cycles may be repeated as desired, *e.g.*, over a period of one or more months, as disclosed herein. After a certain number of cycles, the dosage may be increased to increase the beneficial effect, provided such an increase will not cause

undesirable toxicity effects. Patients may be treated for a minimum number of cycles, as disclosed herein. Complete or partial response may require additional treatment cycles. Treatment may be continued as long as the patient continues to benefit.

J. Example 10

[00254] Clinical studies are conducted to assess the ability of an oral formulation comprising a cytidine analog, such as 5-azacytidine, to treat patients having an ovarian cancer (including, *e.g.*, the ability to stop or reverse the growth of cancer cells in patients having an ovarian cancer). Particular ovarian cancers include, but are not limited to, ovarian epithelial cancer, ovarian germ cell tumors, and ovarian low malignant potential tumors. In certain clinical studies, patients are screened for the presence of a particular type of ovarian cancer prior to administration of the oral formulation. In certain clinical studies, patients with a type of ovarian cancer known or believed to benefit preferentially from cytidine analog (*e.g.*, 5-azacytidine) administration may be enrolled. In certain clinical studies, patients having ovarian cancer are enrolled without screening for particular ovarian cancer types. In certain clinical studies, patients having any type of ovarian cancer are candidates for treatment with an oral formulation provided herein. In certain clinical studies, patients may be currently undergoing additional treatment for ovarian cancer, including, *e.g.*, surgery, chemotherapy, or radiation therapy.

[00255] In certain clinical studies, patients who are administered an oral formulation comprising a cytidine analog (*e.g.*, 5-azacytidine) may also be administered one or more additional therapeutic agents, examples of which are disclosed herein (*e.g.*, carboplatin). The additional therapeutic agent(s) may be administered in the same oral formulation as the cytidine analog, or may be co-administered (*e.g.*, via PO, SC or IV administration) in combination with an oral formulation comprising a cytidine analog. The appropriate amount and dosing schedule for an additional therapeutic agent is determined for a particular patient using methods known in the art.

[00256] An association between gene methylation and ovarian cancer is known in the art. *See, e.g.*, G. Gifford *et al.*, *Clin. Cancer Res.*, 2004, 10:4420-26. Accordingly, in certain clinical studies provided herein, patients are screened prior to enrollment and/or monitored during the trial for DNA or RNA methylation levels, which indicate a potential response to treatment with an oral formulation comprising a cytidine analog (*e.g.*, 5-azacytidine). In certain clinical studies, patients with high levels of DNA methylation (*e.g.*, CpG island methylation) and/or an increased potential for transcriptional silencing of tumor-suppressor

genes may be administered a cytidine analog (*e.g.*, 5-azacytidine) known or believed to prevent or reverse hypermethylation (*e.g.*, by reducing the activity of one or more DNA methyltransferase enzymes). In such studies, patients may also be co-administered one or more additional therapeutic agents known or believed to reduce epigenetic silencing, such as, *e.g.*, compounds that inihibit histone deacetylase enzymes (HDACs), which regulate the acetylation and deacetylation of histone residues that increase or decrease gene expression. *See, e.g.*, J.G. Herman & S.B. Baylin, *N. Engl. J. Med.*, 2003, 349:2042-54; P.A. Jones & S.B. Baylin, *Nature Rev. Gen.*, 2002, 3:415-28. Suitable HDAC inhibitors for co-administration in the clinical studies disclosed herein are known in the art and/or described herein (*e.g.*, entinostat or vorinostat).

[00257] The amount of cytidine analog (*e.g.*, 5-azacytidine) in the oral formulations administered during the clinical studies depends, *e.g.*, on the individual characteristics of the patient, including, *inter alia*, the type, stage, and progression of the patient's ovarian cancer, the patient's age and weight, the patient's prior treatment regimens, and other variables, as known in the art. n certain clinical studies, potential starting doses may be, *e.g.*, about 60 mg, about 120 mg, about 180 mg, about 240 mg, about 300 mg, about 360 mg, about 420 mg, about 480 mg, about 540 mg, about 600 mg, about 660 mg, about 720 mg, about 780 mg, about 840 mg, about 900 mg, about 960 mg, about 1020 mg, or greater than about 1020 mg of the cytidine analog (*e.g.*, 5-azacytidine) daily for a specified time period, *e.g.*, about 1 week, about 1.5 weeks, about 2 weeks, about 2.5 weeks, about 3 weeks, about 3.5 weeks, about 1 month, about 1.5 months, about 2 months, or a longer time period. Other potential starting doses and time periods are disclosed herein. Cycles may be repeated as desired, *e.g.*, over a period of one or more months, as disclosed herein. After a certain number of cycles, the dosage may be increased to increase the beneficial effect, provided such an increase will not cause undesirable toxicity effects. Patients may be treated for a minimum number of cycles, as disclosed herein. Complete or partial response may require additional treatment cycles. Treatment may be continued as long as the patient continues to benefit.

K. Example 11

[00258] Clinical studies are conducted to assess the ability of an oral formulation comprising a cytidine analog, such as 5-azacytidine, to treat patients having a pancreatic cancer (including, *e.g.*, the ability to stop or reverse the growth of cancer cells in patients having pancreatic cancer). In certain clinical studies, patients are screened prior to enrollment for a particular type of pancreatic cancer prior to administration of the oral

formulation. Cellular classifications of pancreatic cancers are known in the art and include, *e.g.*, duct cell carcinoma; acinar cell carcinoma; papillary mucinous carcinoma; signet ring carcinoma; adenosquamous carcinoma; undifferentiated carcinoma; mucinous carcinoma; giant cell carcinoma; mixed type (ductal-endocrine or acinar-endocrine); small cell carcinoma; cystadenocarcinoma (serous and mucinous types); unclassified; pancreatoblastoma; papillary-cystic neoplasm (Frantz tumor); invasive adenocarcinoma associated with cystic mucinous neoplasm or intraductal papillary mucinous neoplasm; mucinous cystic tumor with dysplasia; intraductal papillary mucinous tumor with dysplasia; and pseudopapillary solid tumor. In certain clinical studies, patients are screened prior to enrollment for a particular stage of pancreatic cancer (*e.g.*, the size of the tumor in the pancreas, whether the cancer has spread, and if so, to what parts of the body) prior to administration of the oral formulation. In certain clinical studies, pancreatic cancer patients believed to benefit preferentially from cytidine analog (*e.g.*, 5-azacytidine) administration may be enrolled. In certain clinical studies, patients having pancreatic cancer are enrolled without screening for particular pancreatic cancer types. In certain clinical studies, patients having any type of pancreatic cancer are candidates for treatment with an oral formulation provided herein. In certain clinical studies, patients may be currently undergoing additional treatment for pancreatic cancer, including, *e.g.*, surgery, chemotherapy, or radiation therapy.

[00259] In certain clinical studies, patients who are administered an oral formulation comprising a cytidine analog (*e.g.*, 5-azacytidine) may also be administered one or more additional therapeutic agents, examples of which are disclosed herein (*e.g.*, gemcitabine). The additional therapeutic agent(s) may be administered in the same oral formulation as the cytidine analog, or may be co-administered (*e.g.*, via PO, SC or IV administration) in combination with an oral formulation comprising a cytidine analog. The appropriate amount and dosing schedule for an additional therapeutic agent is determined for a particular patient using methods known in the art.

[00260] In certain clinical studies provided herein, patients are screened prior to enrollment and/or monitored during the trial for DNA or RNA methylation levels, which indicate a potential response to treatment with an oral formulation comprising a cytidine analog (*e.g.*, 5-azacytidine). In certain clinical studies, patients with high levels of DNA methylation (*e.g.*, CpG island methylation) and/or an increased potential for transcriptional silencing of tumor-suppressor genes may be administered a cytidine analog (*e.g.*, 5-azacytidine) known or believed to prevent or reverse hypermethylation (*e.g.*, by reducing the

activity of one or more DNA methyltransferase enzymes). In such studies, patients may also be co-administered one or more additional therapeutic agents known or believed to reduce epigenetic silencing, such as, *e.g.*, compounds that inihibit histone deacetylase enzymes (HDACs), which regulate the acetylation and deacetylation of histone residues that increase or decrease gene expression. *See, e.g.*, J.G. Herman & S.B. Baylin, *N. Engl. J. Med.*, 2003, 349:2042-54; P.A. Jones & S.B. Baylin, *Nature Rev. Gen.*, 2002, 3:415-28. Suitable HDAC inhibitors for co-administration in the clinical studies disclosed herein are known in the art and/or described herein (*e.g.*, entinostat or vorinostat).

[00261] The amount of cytidine analog (*e.g.*, 5-azacytidine) in the oral formulations administered during the clinical studies depends, *e.g.*, on the individual characteristics of the patient, including, *inter alia*, the type, stage, and progression of the patient's pancreatic cancer, the patient's age and weight, the patient's prior treatment regimens, and other variables, as known in the art. In certain clinical studies, potential starting doses may be, *e.g.*, about 60 mg, about 120 mg, about 180 mg, about 240 mg, about 300 mg, about 360 mg, about 420 mg, about 480 mg, about 540 mg, about 600 mg, about 660 mg, about 720 mg, about 780 mg, about 840 mg, about 900 mg, about 960 mg, about 1020 mg, or greater than about 1020 mg of the cytidine analog (*e.g.*, 5-azacytidine) daily for a specified time period, *e.g.*, about 1 week, about 1.5 weeks, about 2 weeks, about 2.5 weeks, about 3 weeks, about 3.5 weeks, about 1 month, about 1.5 months, about 2 months, or a longer time period. Other potential starting doses and time periods are disclosed herein. Cycles may be repeated as desired, *e.g.*, over a period of one or more months, as disclosed herein. After a certain number of cycles, the dosage may be increased to increase the beneficial effect, provided such an increase will not cause undesirable toxicity effects. Patients may be treated for a minimum number of cycles, as disclosed herein. Complete or partial response may require additional treatment cycles. Treatment may be continued as long as the patient continues to benefit.

L. Example 12

[00262] Clinical studies are conducted to assess the ability of an oral formulation comprising a cytidine analog, such as 5-azacytidine, to treat patients having a colorectal cancer (including, *e.g.*, the ability to stop or reverse the growth of cancer cells in patients having a colorectal cancer). In certain clinical studies, patients are screened prior to enrollment for a particular type of colorectal cancer prior to administration of the oral formulation. Histologic types of colon cancers are known in the art and include, *e.g.*,

adenocarcinoma; mucinous (colloid) adenocarcinoma; signet ring adenocarcinoma; scirrhous tumors; and neuroendocrine tumors. The World Health Organization classification of tumors of the colon and rectum include (1) Epithelial Tumors, which include: Adenoma (*e.g.*, tubular, villous, tubulovillous, and serrated); Intraepithelial neoplasia (dysplasia) associated with chronic inflammatory diseases (*e.g.*, low-grade glandular intraepithelial neoplasia and high-grade glandular intraepithelial neoplasia); Carcinoma (*e.g.*, adenocarcinoma, mucinous adenocarcinoma, signet-ring cell carcinoma, small cell carcinoma, adenosquamous carcinoma, medullary carcinoma, and undifferentiated carcinoma); Carcinoid (well-differentiated neuroendocrine neoplasm) (*e.g.*, enterochromaffin (EC)-cell, serotonin-producing neoplasm, L-cell, glucagon-like peptide and pancreatic polypeptide/peptide YY (PYY)-producing tumor, and others); and Mixed carcinoma-adenocarcinoma; and (2) Nonepithelial Tumors, which include: Lipoma; Leiomyoma; Gastrointestinal stromal tumor; Leiomyosarcoma; Angiosarcoma; Kaposi sarcoma; Melanoma; and others; as well as Malignant lymphomas (*e.g.*, marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue type, mantle cell lymphoma, diffuse large B-cell lymphoma, Burkitt lymphoma, and Burkitt-like/atypical Burkitt lymphoma. In certain clinical studies, patients are screened prior to enrollment for a particular stage of colorectal cancer (*e.g.*, the size of the tumor in the colon or rectum, whether the cancer has spread, and if so, to what parts of the body) prior to administration of the oral formulation. In certain clinical studies, colorectal cancer patients believed to benefit preferentially from cytidine analog (*e.g.*, 5-azacytidine) administration may be enrolled. In certain clinical studies, patients having a colorectal cancer are enrolled without screening for particular colorectal cancer types. In certain clinical studies, patients having any type of colorectal cancer are candidates for treatment with an oral formulation provided herein. In certain clinical studies, patients may be currently undergoing additional treatment for colorectal cancer, including, *e.g.*, surgery, chemotherapy, or radiation therapy.

[00263] In certain clinical studies, patients who are administered an oral formulation comprising a cytidine analog (*e.g.*, 5-azacytidine) may also be administered one or more additional therapeutic agents, examples of which are disclosed herein. The additional therapeutic agent(s) may be administered in the same oral formulation as the cytidine analog, or may be co-administered (*e.g.*, via PO, SC or IV administration) in combination with an oral formulation comprising a cytidine analog. The appropriate amount and dosing schedule for an additional therapeutic agent is determined for a particular patient using methods known in the art.

[00264] An association between gene methylation and colorectal cancer is known in the art. *See, e.g.*, A.M. Jubb *et al.*, *J. Pathol.*, 2001, 195:111-134. Accordingly, in certain clinical studies provided herein, patients are screened prior to enrollment and/or monitored during the trial for DNA or RNA methylation levels, which indicate a potential response to treatment with an oral formulation comprising a cytidine analog (*e.g.*, 5-azacytidine). In certain clinical studies, patients with high levels of DNA methylation (*e.g.*, CpG island methylation) and/or an increased potential for transcriptional silencing of tumor-suppressor genes may be administered a cytidine analog (*e.g.*, 5-azacytidine) known or believed to prevent or reverse hypermethylation (*e.g.*, by reducing the activity of one or more DNA methyltransferase enzymes). In such studies, patients may also be co-administered one or more additional therapeutic agents known or believed to reduce epigenetic silencing, such as, *e.g.*, compounds that inihibit histone deacetylase enzymes (HDACs), which regulate the acetylation and deacetylation of histone residues that increase or decrease gene expression. *See, e.g.*, J.G. Herman & S.B. Baylin, *N. Engl. J. Med.*, 2003, 349:2042-54; P.A. Jones & S.B. Baylin, *Nature Rev. Gen.*, 2002, 3:415-28. Suitable HDAC inhibitors for co-administration in the clinical studies disclosed herein are known in the art and/or described herein (*e.g.*, entinostat or vorinostat).

[00265] The amount of cytidine analog (*e.g.*, 5-azacytidine) in the oral formulations administered during the clinical studies depends, *e.g.*, on the individual characteristics of the patient, including, *inter alia*, the type, stage, and progression of the patient's colorectal cancer, the patient's age and weight, the patient's prior treatment regimens, and other variables, as known in the art. In certain clinical studies, potential starting doses may be, *e.g.*, about 60 mg, about 120 mg, about 180 mg, about 240 mg, about 300 mg, about 360 mg, about 420 mg, about 480 mg, about 540 mg, about 600 mg, about 660 mg, about 720 mg, about 780 mg, about 840 mg, about 900 mg, about 960 mg, about 1020 mg, or greater than about 1020 mg of the cytidine analog (*e.g.*, 5-azacytidine) daily for a specified time period, *e.g.*, about 1 week, about 1.5 weeks, about 2 weeks, about 2.5 weeks, about 3 weeks, about 3.5 weeks, about 1 month, about 1.5 months, about 2 months, or a longer time period. Other potential starting doses and time periods are disclosed herein. After a certain number of cycles, the dosage may be increased to increase the beneficial effect, provided such an increase will not cause undesirable toxicity effects. Patients may be treated for a minimum number of cycles, as disclosed herein. Complete or partial response may require additional treatment cycles. Treatment may be continued as long as the patient continues to benefit.

[00266] The present disclosure has been described in connection with certain embodiments and examples; however, unless otherwise indicated, the claimed invention should not be unduly limited to such specific embodiments and examples.

WHAT IS CLAIMED IS:

1. A pharmaceutical composition for oral administration comprising a therapeutically effective amount of 5-azacytidine, wherein the composition releases the 5-azacytidine substantially in the stomach following oral administration to a subject.

2. The composition of claim 1, which is an immediate release composition.

3. The composition of claim 1, which is non-enteric-coated.

4. The composition of claim 1, which is a tablet.

5. The composition of claim 1, which is a capsule.

6. The composition of claim 1, which further comprises an excipient selected from mannitol, microcrystalline cellulose, crospovidone, and magnesium stearate.

7. The composition of claim 1, which further comprises a permeation enhancer.

8. The composition of claim 7, wherein the permeation enhancer is d-alpha-tocopheryl polyethylene glycol 1000 succinate.

9. The composition of claim 8, wherein the d-alpha-tocopheryl polyethylene glycol 1000 succinate is present in the formulation at about 2% by weight relative to the total weight of the formulation.

10. The composition of claim 1, which is essentially free of a cytidine deaminase inhibitor.

11. The composition of claim 1, which is essentially free of tetrahydrouridine.

12. The composition of claim 1, which further comprises an additional therapeutic agent.

13. The composition of claim 1, wherein the amount of 5-azacytidine is at least about 40 mg.

14. The composition of claim 1, wherein the amount of 5-azacytidine is at least about 400 mg.

15. The composition of claim 1, wherein the amount of 5-azacytidine is at least about 1000 mg.

16. The composition of claim 1, which achieves an area-under-the-curve value of at least about 200 ng-hr/mL following oral administration to a subject.

17. The composition of claim 1, which achieves an area-under-the-curve value of at least about 400 ng-hr/mL following oral administration to a subject.

18. The composition of claim 1, which achieves a maximum plasma concentration of at least about 100 ng/mL following oral administration to a subject.

19. The composition of claim 1, which achieves a maximum plasma concentration of at least about 200 ng/mL following oral administration to a subject.

20. The composition of claim 1, which achieves a time to maximum plasma concentration of less than about 180 minutes following oral administration to a subject.

21. The composition of claim 1, which achieves a time to maximum plasma concentration of less than about 90 minutes following oral administration to a subject.

22. The composition of claim 1, which achieves a time to maximum plasma concentration of less than about 60 minutes following oral administration to a subject.

23. A pharmaceutical composition for oral administration comprising a therapeutically effective amount of 5-azacytidine, which releases the 5-azacytidine substantially in the stomach and achieves an area-under-the-curve value of at least about 200 ng-hr/mL following oral administration.

24. A pharmaceutical composition for oral administration comprising a therapeutically effective amount of 5-azacytidine, which releases the 5-azacytidine substantially in the stomach and achieves an area-under-the-curve value of at least about 400 ng-hr/mL following oral administration.

25. A pharmaceutical composition for oral administration comprising a therapeutically effective amount of 5-azacytidine, which releases the 5-azacytidine substantially in the stomach and achieves a maximum plasma concentration of at least about 100 ng/mL following oral administration.

26. A pharmaceutical composition for oral administration comprising a therapeutically effective amount of 5-azacytidine, which releases the 5-azacytidine substantially in the stomach and achieves a maximum plasma concentration of at least about 200 ng/mL following oral administration.

27. A pharmaceutical composition for oral administration comprising a therapeutically effective amount of 5-azacytidine, which releases the 5-azacytidine substantially in the stomach and achieves a time to maximum plasma concentration of less than about 180 minutes following oral administration.

28. A pharmaceutical composition for oral administration comprising a therapeutically effective amount of 5-azacytidine, which releases the 5-azacytidine substantially in the stomach and achieves a time to maximum plasma concentration of less than about 90 minutes following oral administration.

29. The composition of any one of claims 23-28, which is a single unit dosage form.

30. The composition of claim 29, which is non-enteric-coated.

31. The composition of claim 29, which is a tablet.

32. The composition of claim 29, which is a capsule.

33. A method for treating a subject having a disease associated with abnormal cell proliferation, comprising orally administering to the subject a pharmaceutical composition comprising a therapeutically effective amount of 5-azacytidine, wherein the composition releases the 5-azacytidine substantially in the stomach following oral administration to the subject.

34. The method of claim 33, wherein the disease is myelodysplastic syndrome.

35. The method of claim 33, wherein the disease is acute myelogenous leukemia.

36. The method of claim 33, wherein the disease is non-small-cell lung cancer.

37. The method of claim 33, wherein the disease is ovarian cancer.

38. The method of claim 33, wherein the disease is pancreatic cancer.

39. The method of claim 33, wherein the disease is colorectal cancer.

40. The method of claim 33, wherein the method further comprises co-administering to the subject in need thereof an additional therapeutic agent.

41. The method of claim 33, wherein the composition is an immediate release composition.

42. The method of claim 33, wherein the composition further comprises a permeation enhancer.

43. The method of claim 42, wherein the permeation enhancer is d-alpha-tocopheryl polyethylene glycol 1000 succinate.

44. The method of claim 43, wherein the d-alpha-tocopheryl polyethylene glycol 1000 succinate is present in the formulation at about 2% by weight relative to the total weight of the formulation.

45. The method of claim 33, wherein the method further comprises not co-administering a cytidine deaminase inhibitor with the cytidine analog.

46. The method of claim 33, wherein the composition is a single unit dosage form.

47. The method of claim 33, wherein the composition is non-enteric-coated.

48. The method of claim 33, wherein the composition is a tablet.

49. The method of claim 33, wherein the composition is a capsule.

50. The method of claim 33, wherein the composition further comprises an excipient selected from mannitol, microcrystalline cellulose, crospovidone, and magnesium stearate.

51. The method of claim 33, wherein the amount of 5-azacytidine is at least about 40 mg.

52. The method of claim 33, wherein the amount of 5-azacytidine is at least about 400 mg.

53. The method of claim 33, wherein the amount of 5-azacytidine is at least about 1000 mg.

54. The method of claim 33, which achieves an area-under-the-curve value of at least about 200 ng-hr/mL following oral administration to the subject.

55. The method of claim 33, which achieves an area-under-the-curve value of at least about 400 ng-hr/mL following oral administration to the subject.

56. The method of claim 33, which achieves a maximum plasma concentration of at least about 100 ng/mL following oral administration to the subject.

57. The method of claim 33, which achieves a maximum plasma concentration of at least about 200 ng/mL following oral administration to the subject.

58. The method of claim 33, which achieves a time to maximum plasma concentration of less than about 180 minutes following oral administration to the subject.

59. The method of claim 33, which achieves a time to maximum plasma concentration of less than about 90 minutes following oral administration to the subject.

60. A pharmaceutical composition comprising a therapeutically effective amount of 5-azacytidine, wherein the composition is for treating a disease or disorder associated with abnormal cell proliferation, wherein the composition is prepared for oral administration, and wherein the composition is prepared for release of the 5-azacytidine substantially in the stomach.

61. The pharmaceutical composition of claim 60, wherein the amount of 5-azacytidine is about 40 mg, about 400 mg, or about 1000 mg.

62. The pharmaceutical composition of claim 60, wherein the composition is prepared to achieve an area-under-the-curve value of at least about 200 ng-hr/mL or 400 ng-hr/mL following oral administration.

63. The pharmaceutical composition of claim 60, wherein the composition is prepared to achieve a maximum plasma concentration of at least about 100 ng/mL or 200 ng/mL following oral administration.

64. The pharmaceutical composition of claim 60, wherein the composition is prepared to achieve a time to maximum plasma concentration of less than about 60 minutes or 90 minutes after being administered.

65. The pharmaceutical composition of any one of claims 60 to 64, wherein the composition is prepared in the form of an immediate release composition.

66. The pharmaceutical composition of any one of claims 60 to 64, wherein the composition is prepared for oral administration in combination with an additional therapeutic agent.

67. The pharmaceutical composition of any one of claims 60 to 64, wherein the disease or disorder is myelodysplastic syndrome or acute myelogenous leukemia.

68. The pharmaceutical composition of any one of claims 60 to 64, wherein the composition is a single unit dosage form.

69. The pharmaceutical composition of any one of claims 60 to 64, wherein the composition is a tablet or a capsule.

70. The pharmaceutical composition of any one of claims 60 to 64, wherein the composition further comprises an excipient selected from mannitol, microcrystalline cellulose, crospovidone, and magnesium stearate.

71. Use of 5-azacytidine for the preparation of a pharmaceutical composition for treating a disease associated with abnormal cell proliferation, wherein the composition is prepared for oral administration, and wherein the composition is prepared for release of the 5-azacytidine substantially in the stomach.

72. The use of claim 71, wherein the disease is myelodysplastic syndrome or acute myelogenous leukemia.

73. The use of claim 71 or 72, wherein the amount of 5-azacytidine is about 40 mg, about 400 mg, or about 1000 mg.

74. The use of any one of claims 71 to 73, wherein the composition is prepared for immediate release.

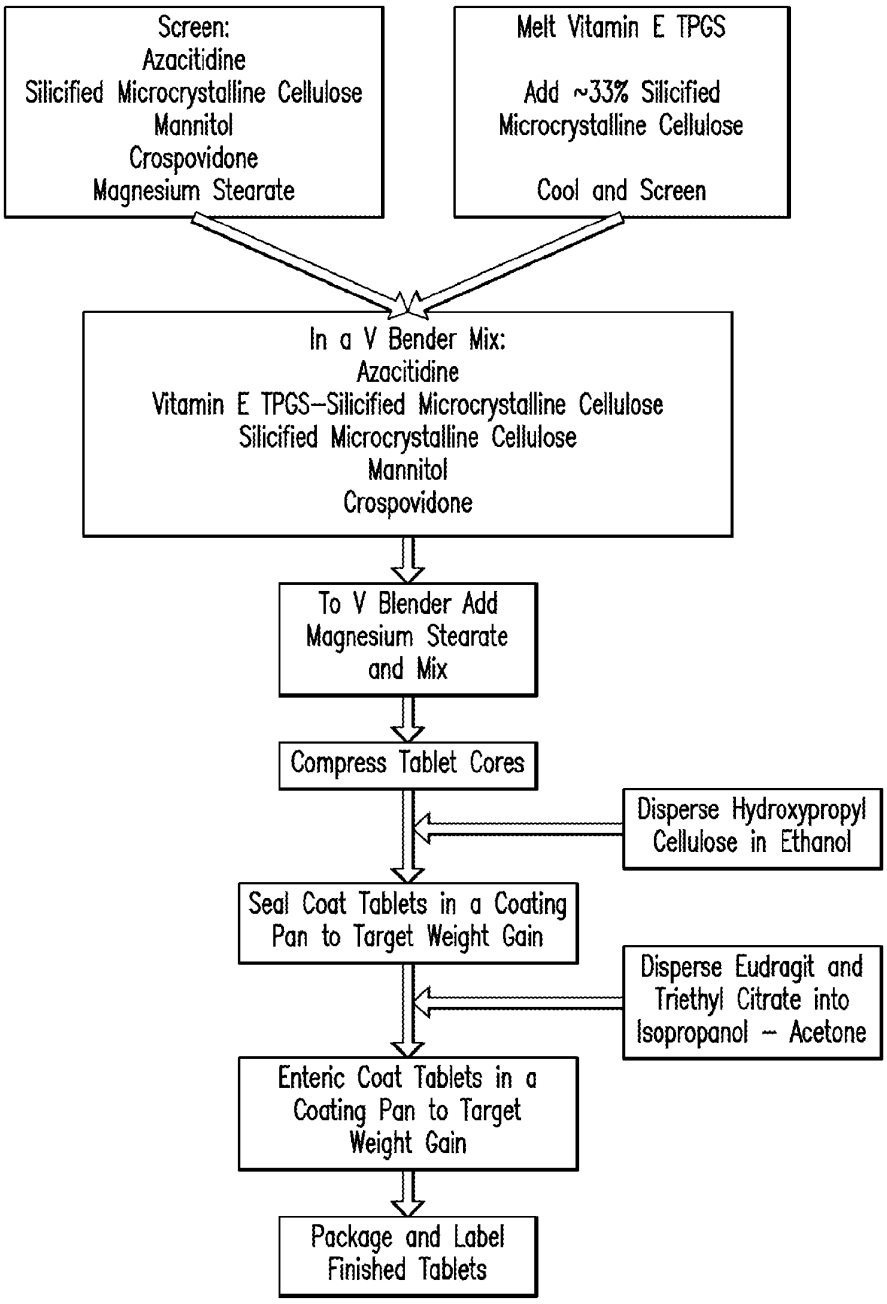


FIG. 1

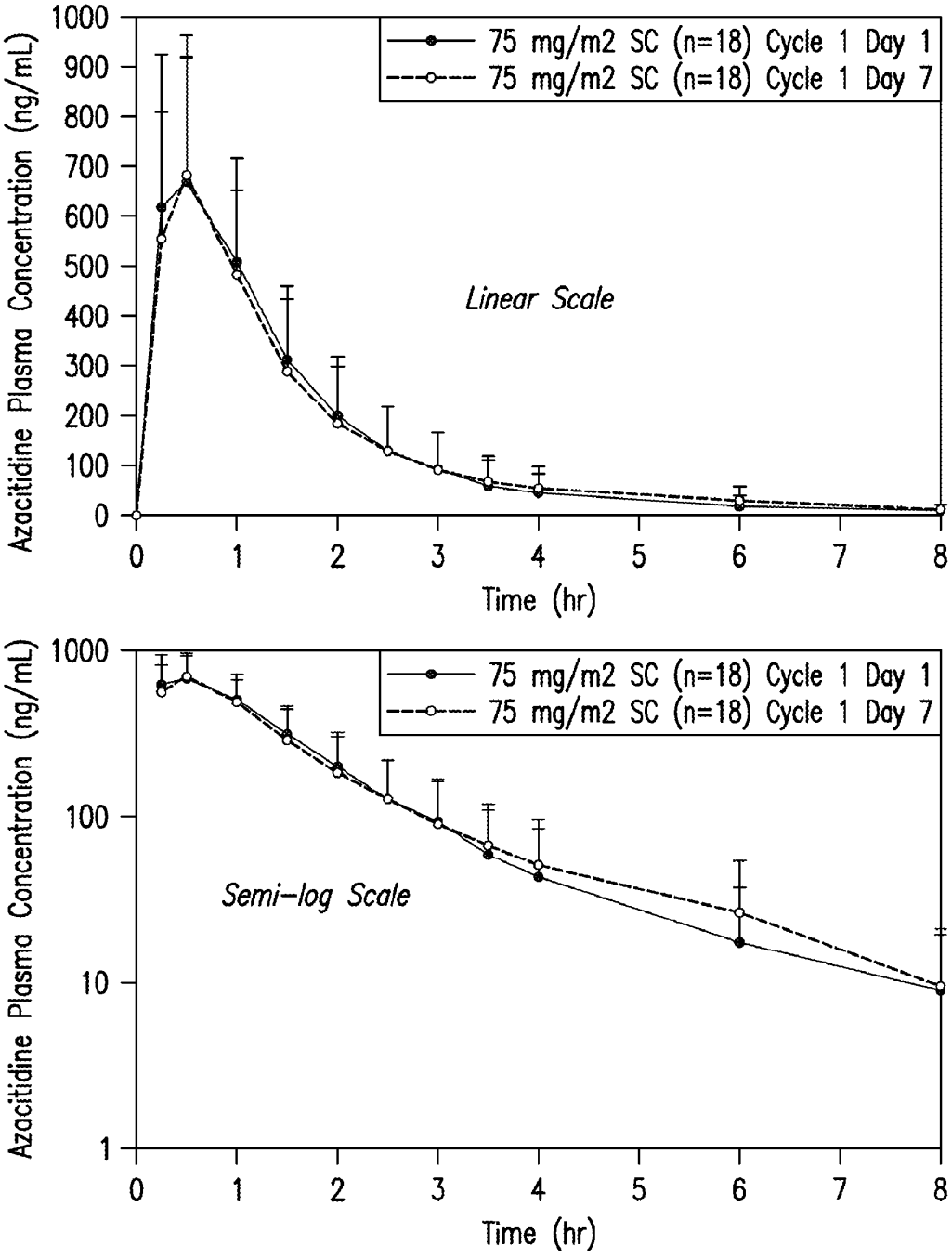


FIG.2

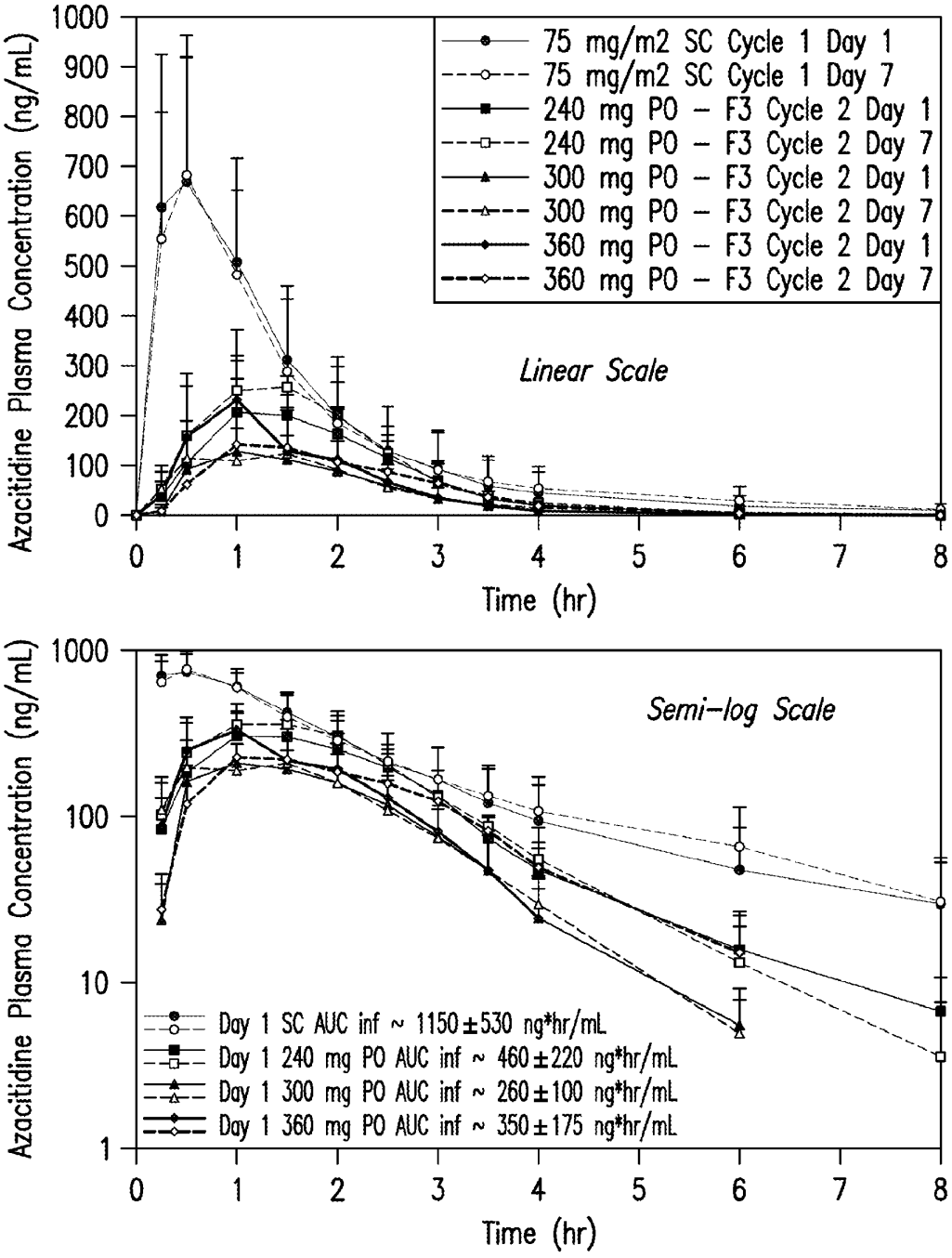


FIG.3

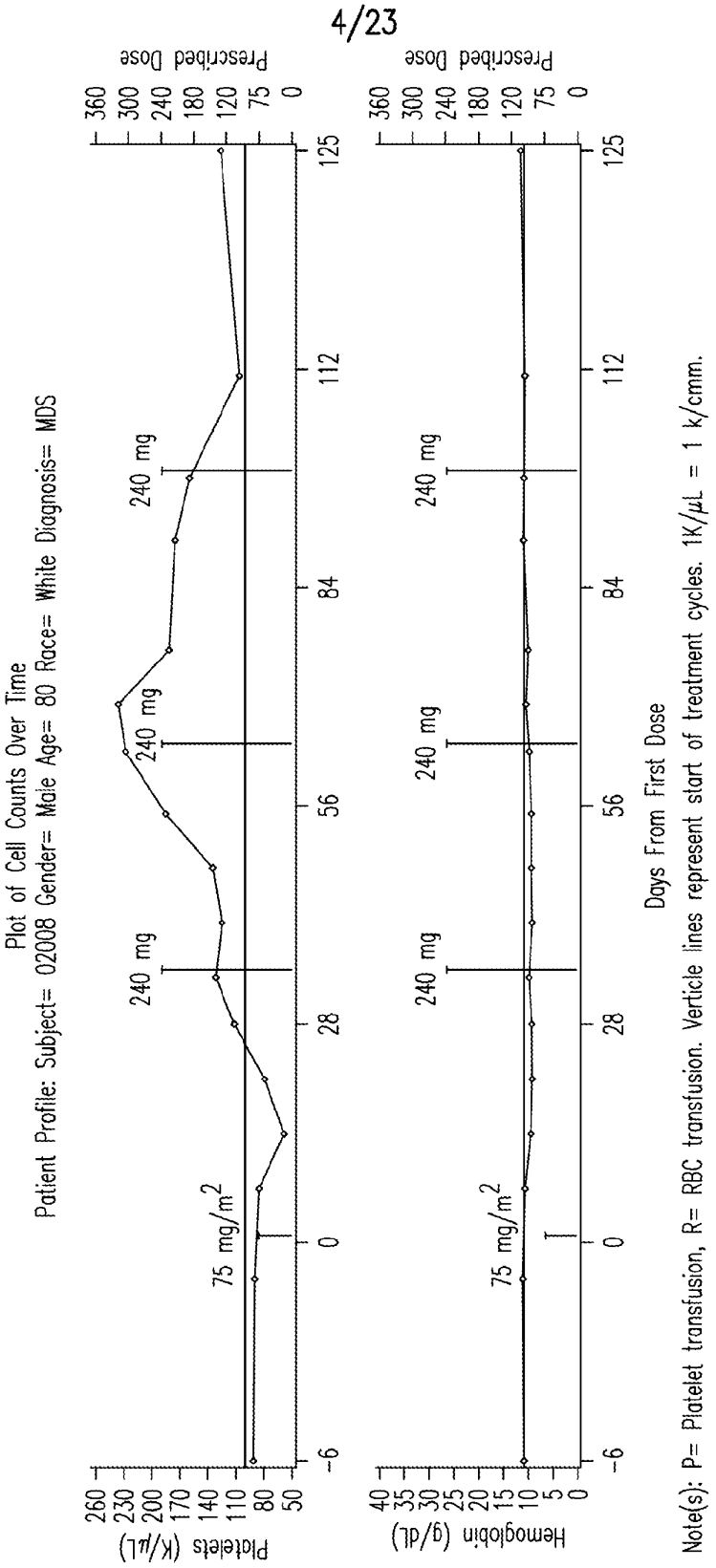


FIG.4A

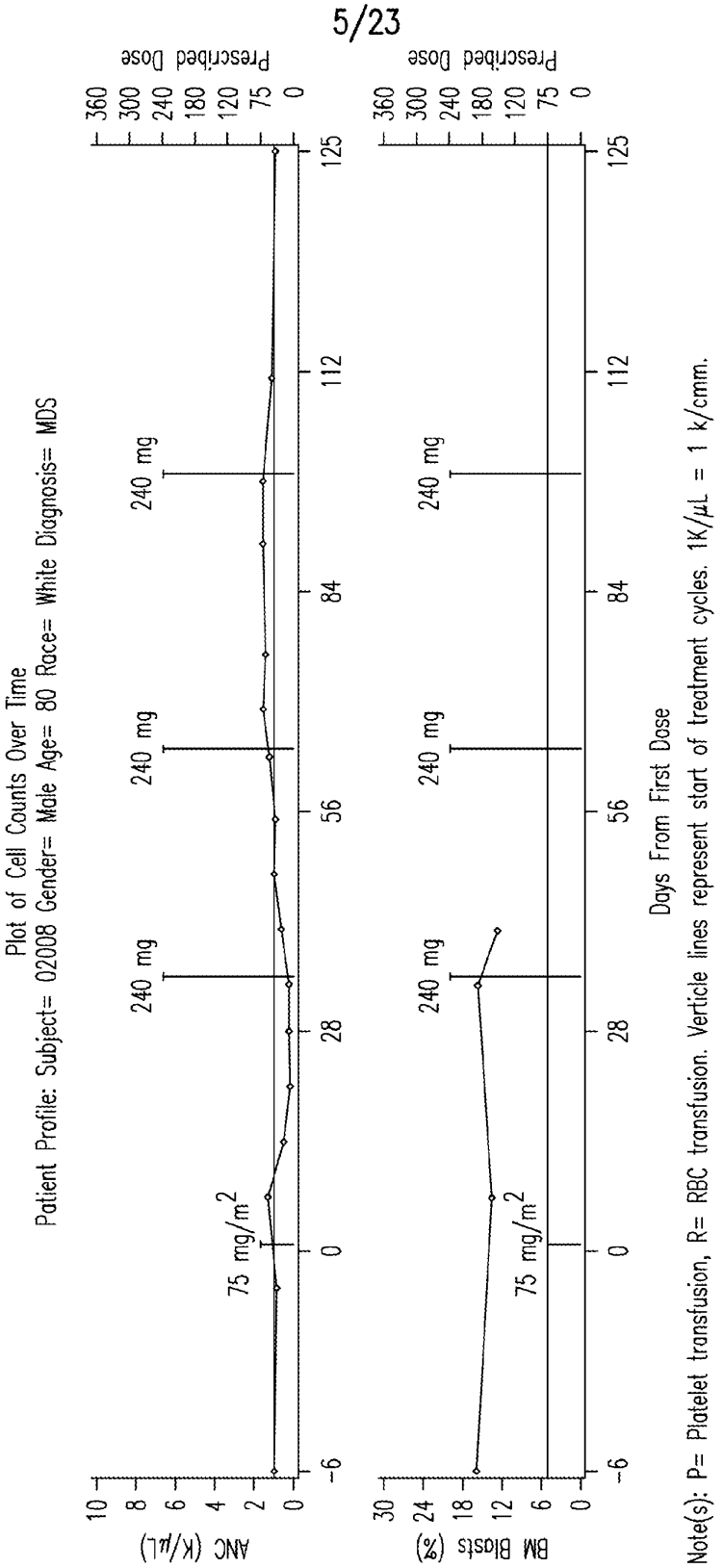


FIG.4B

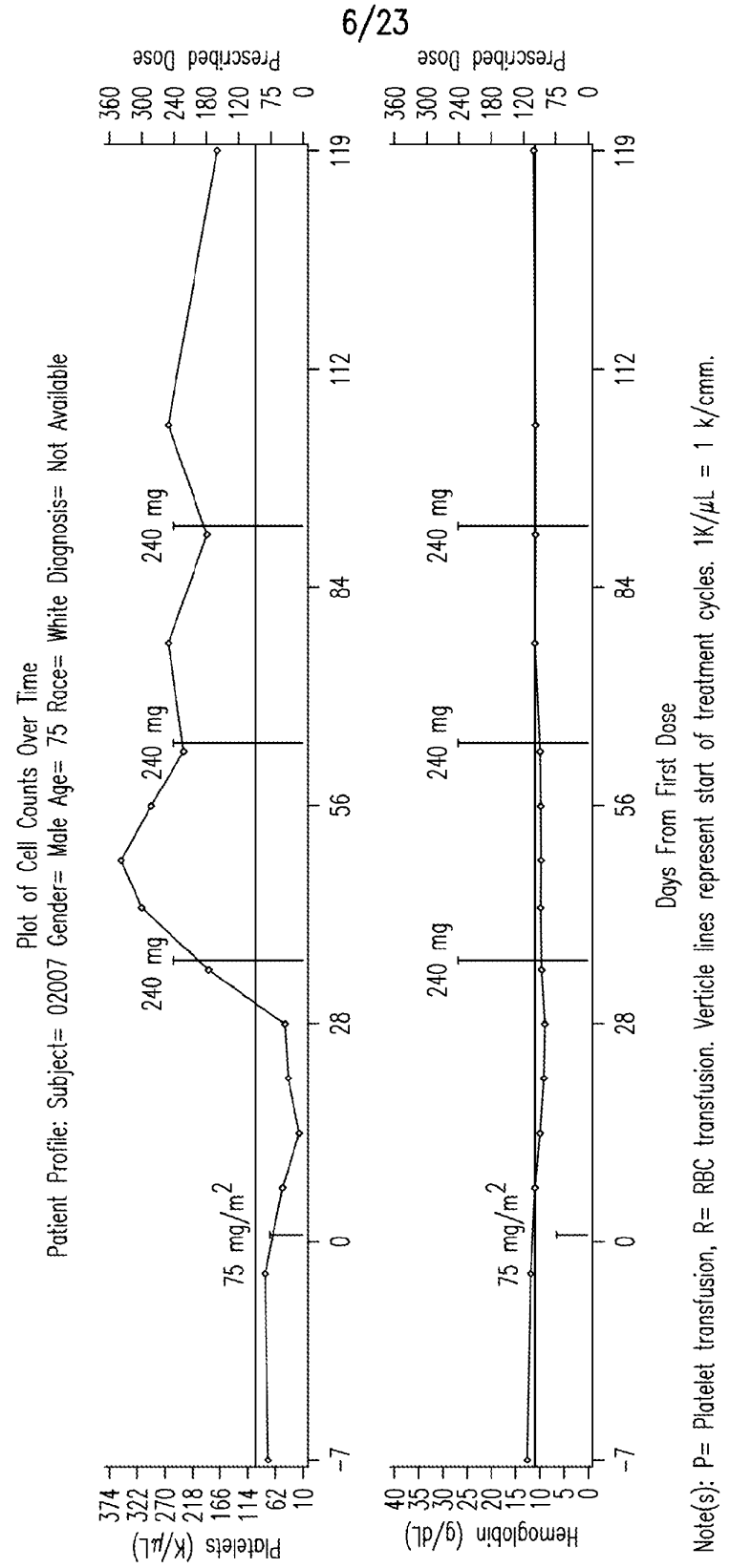


FIG.5A

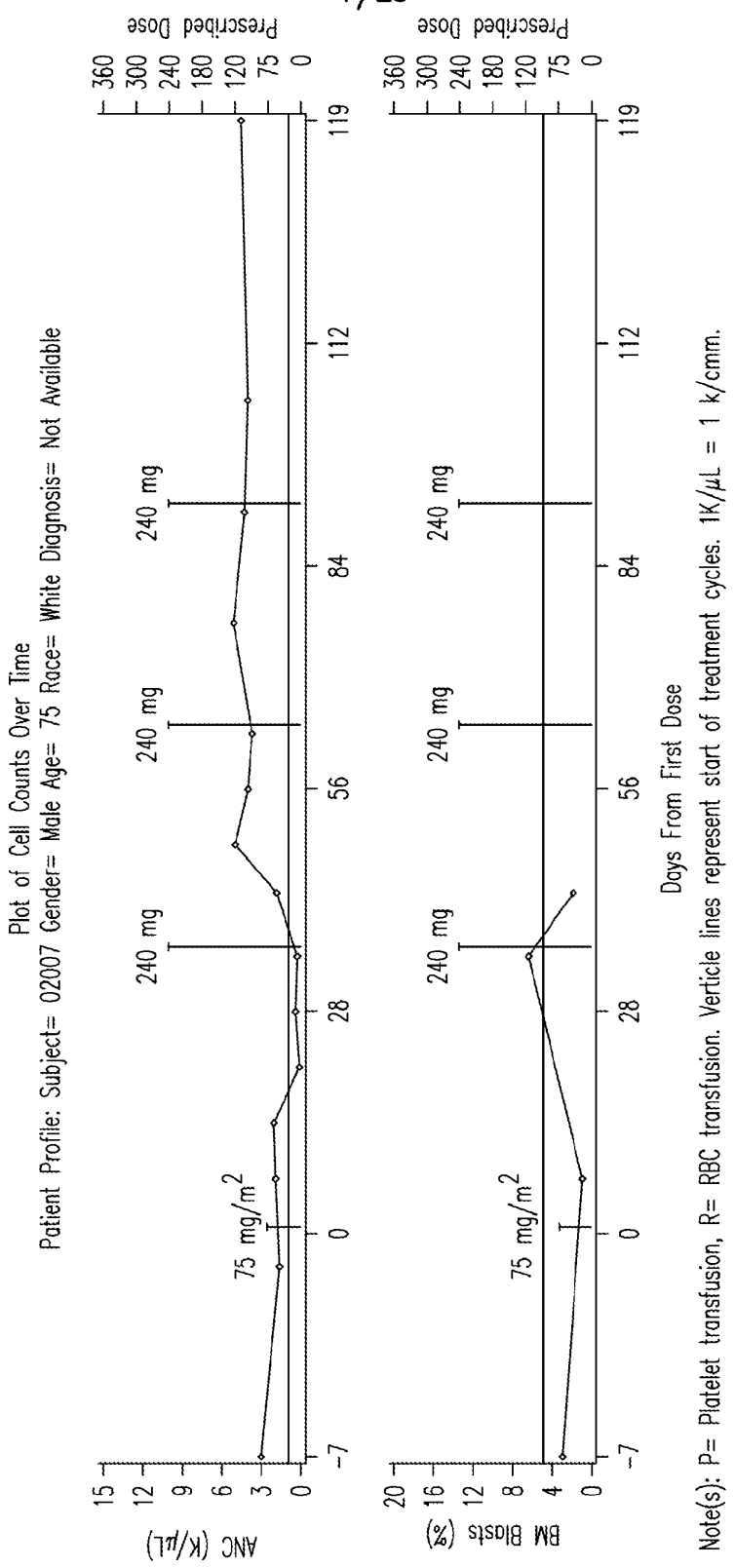


FIG.5B

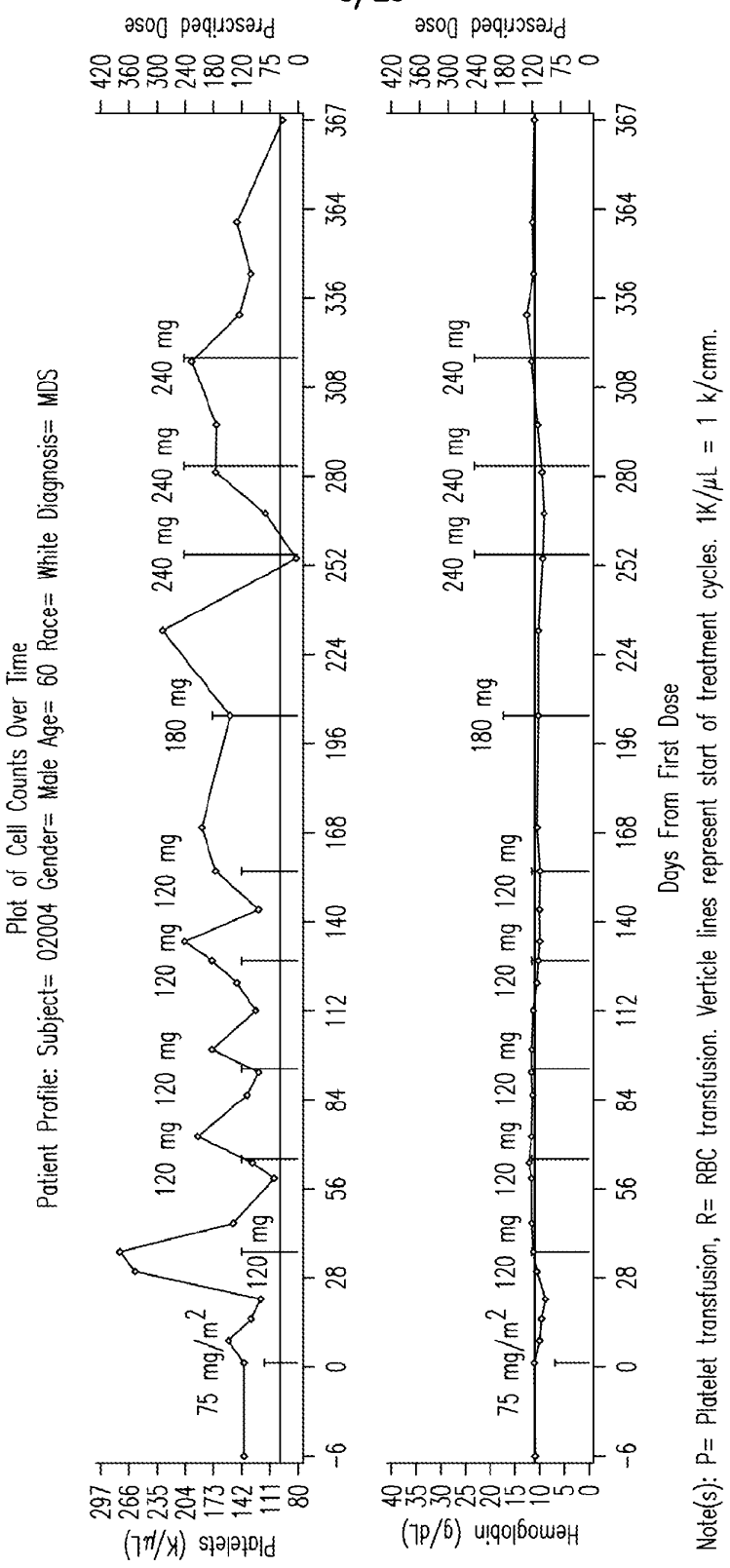


FIG.6A

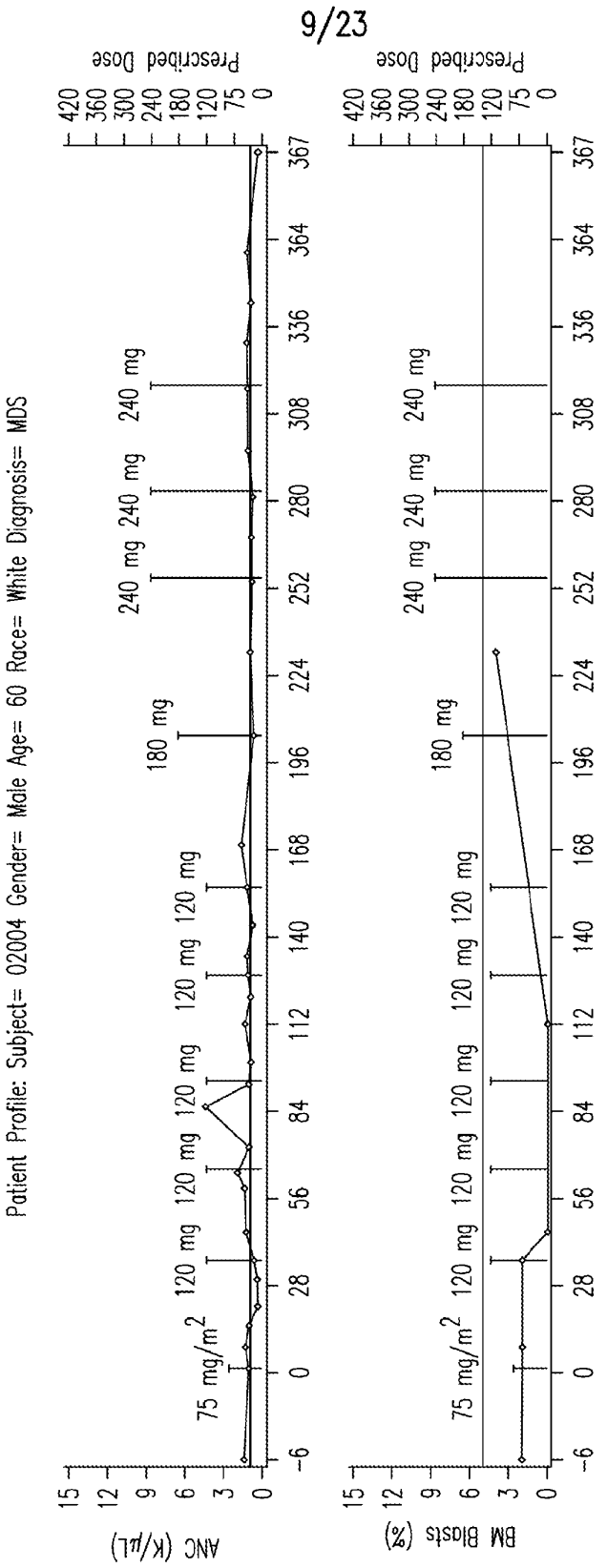


FIG.6B

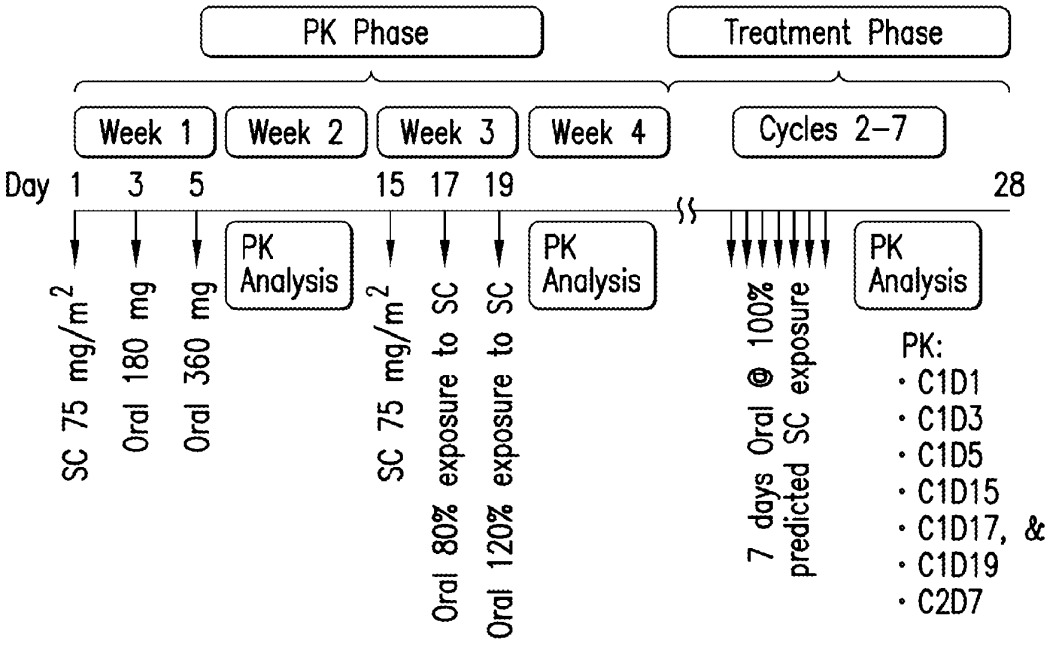


FIG.7

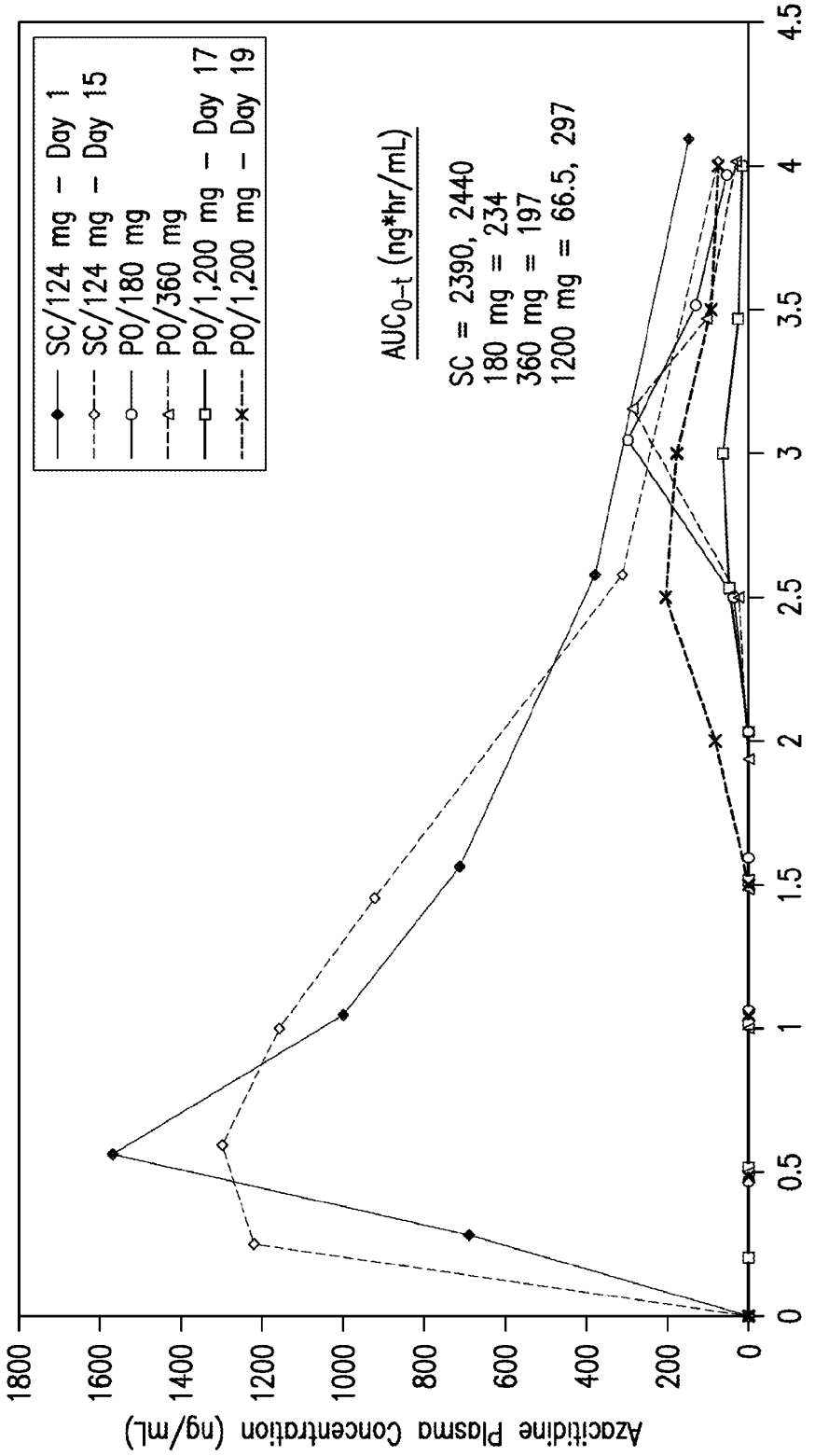


FIG.8

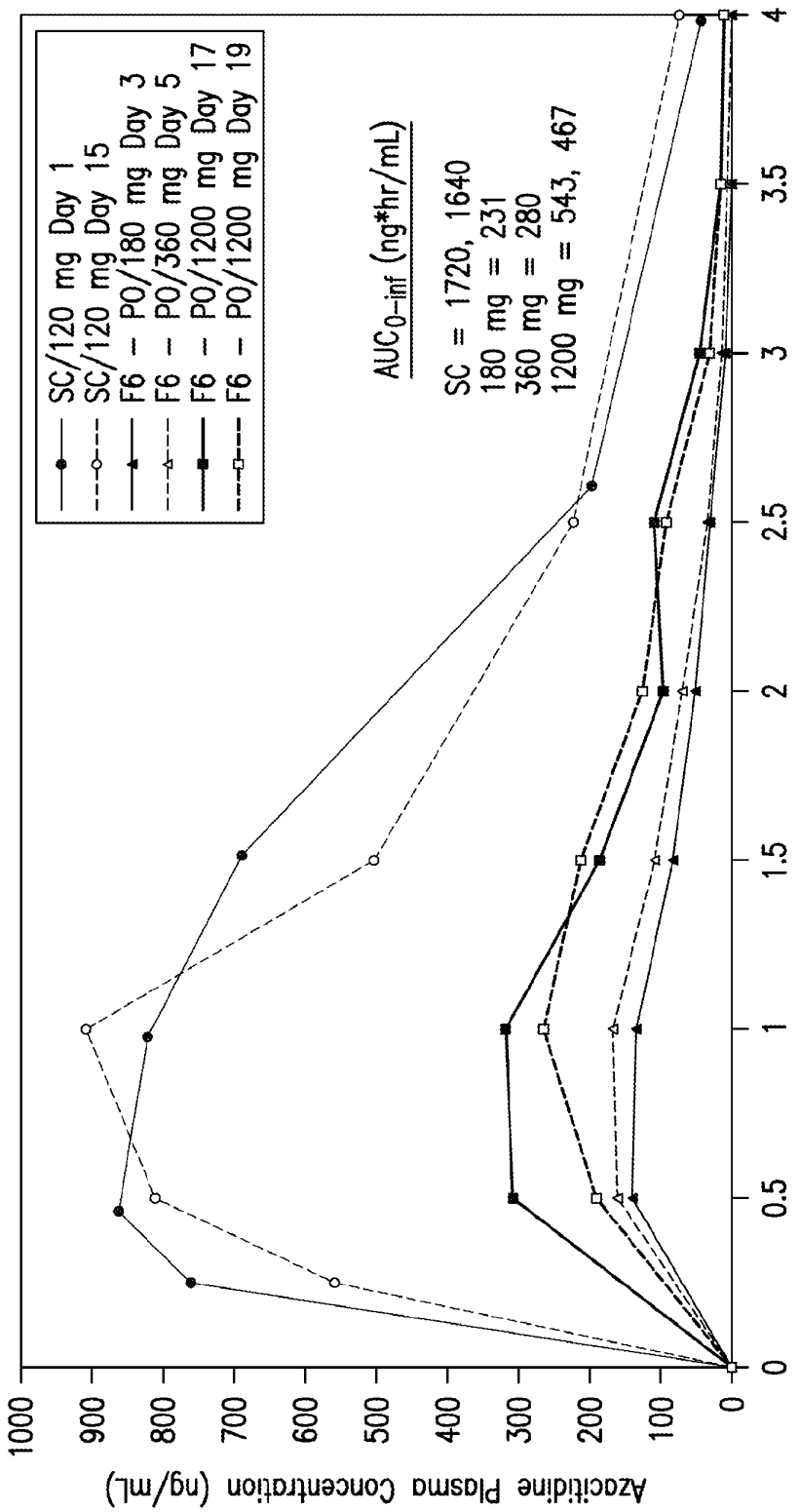


FIG.9

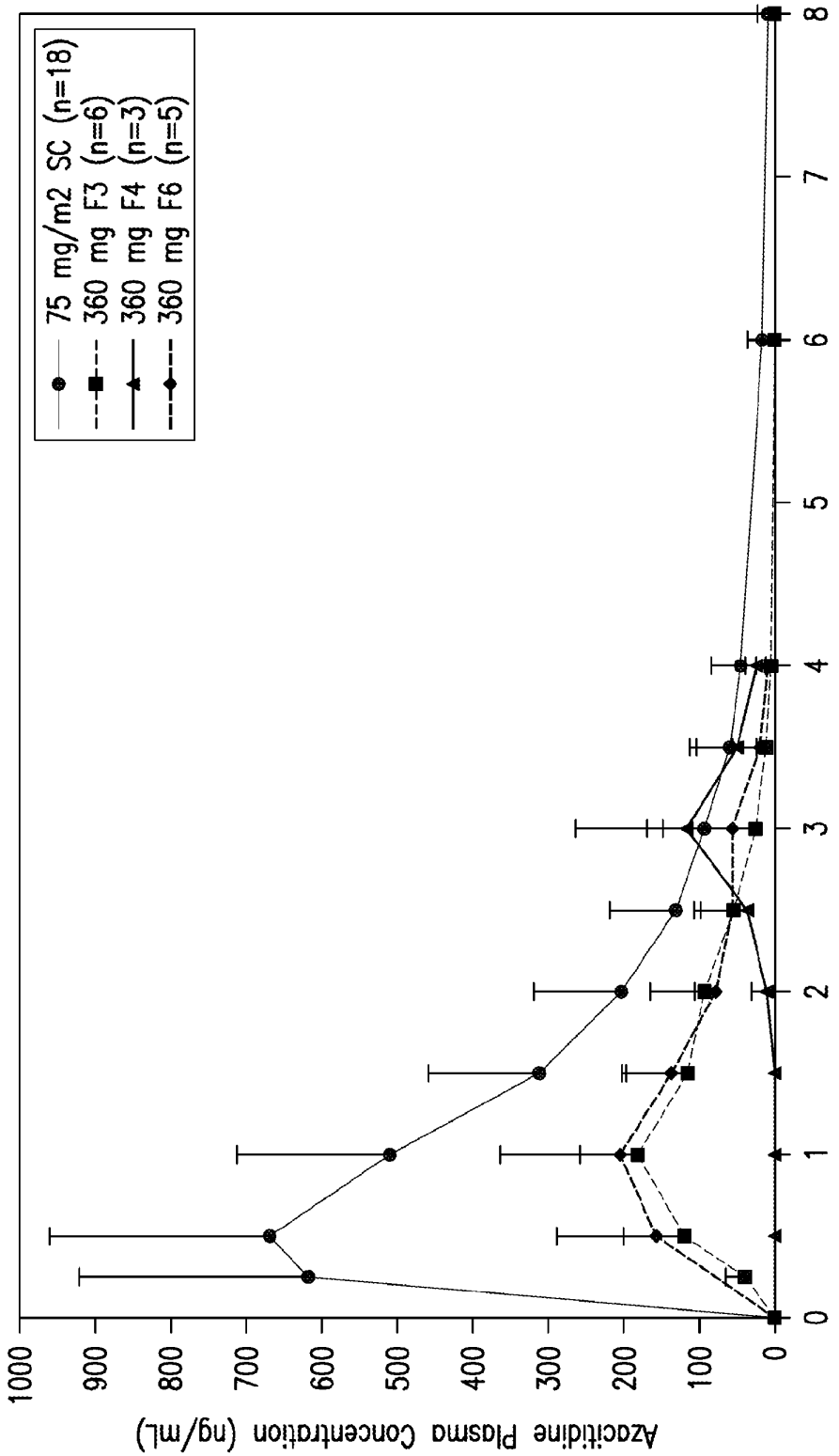


FIG. 10

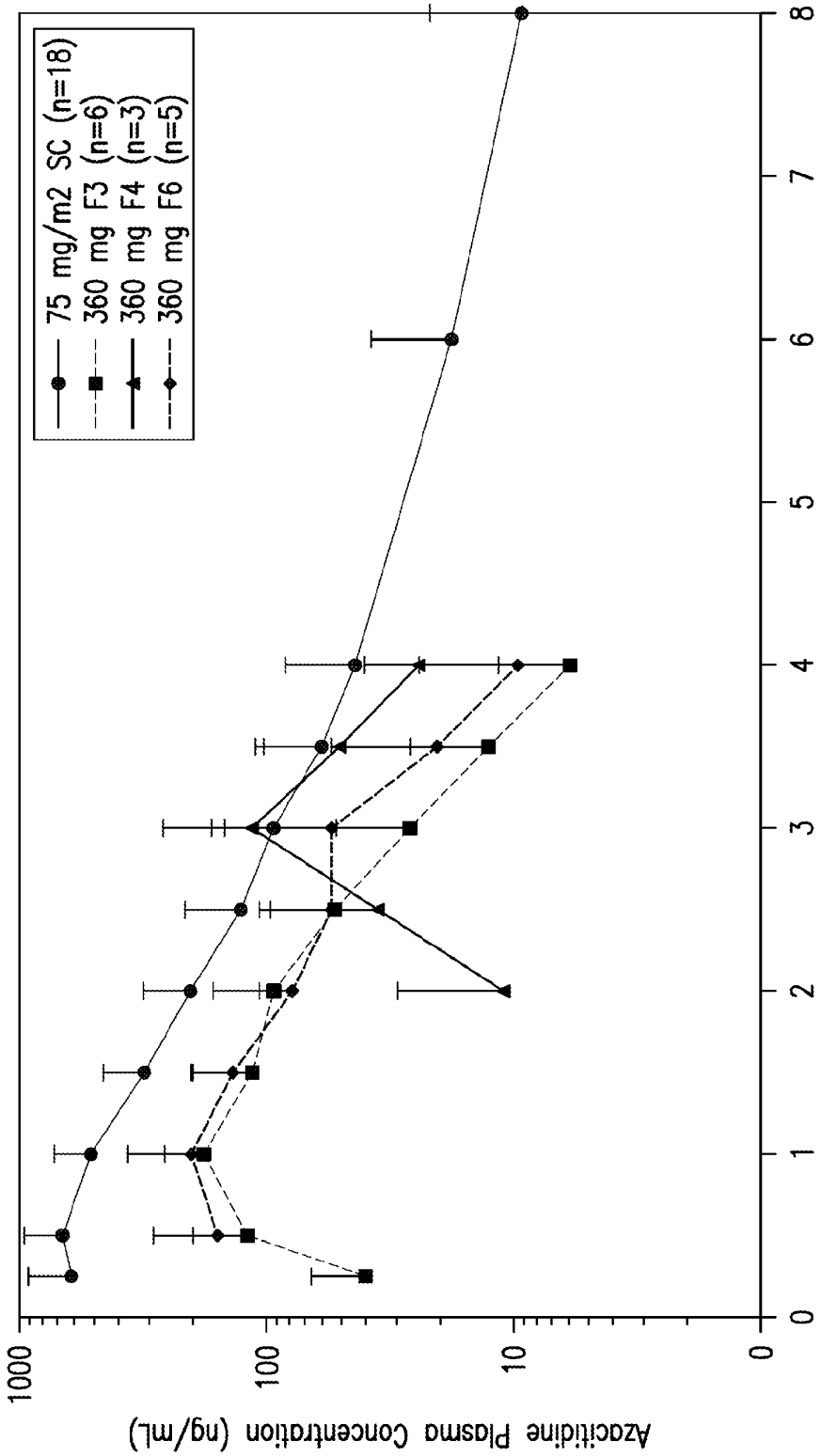


FIG.11

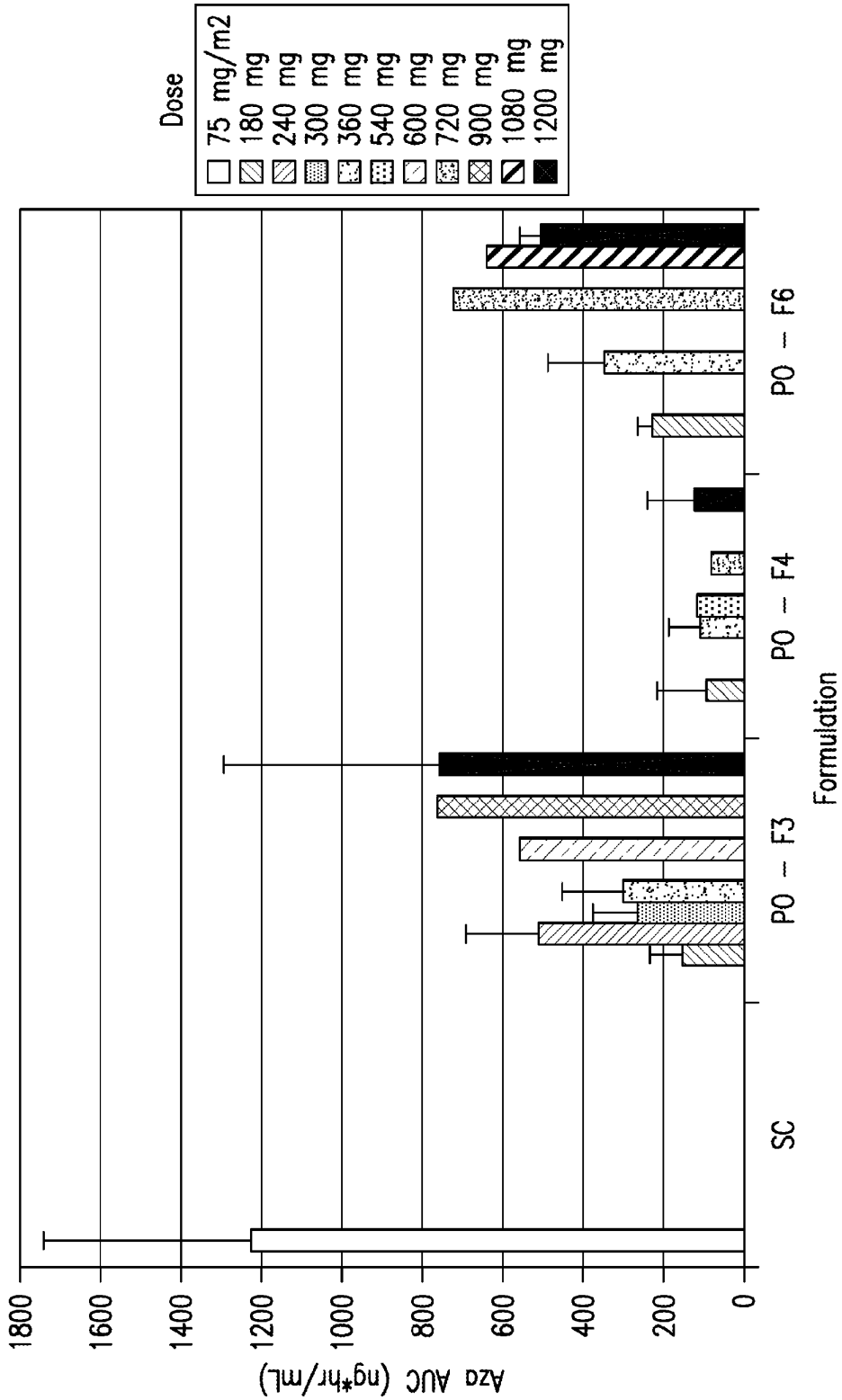


FIG. 12

FIG. 13

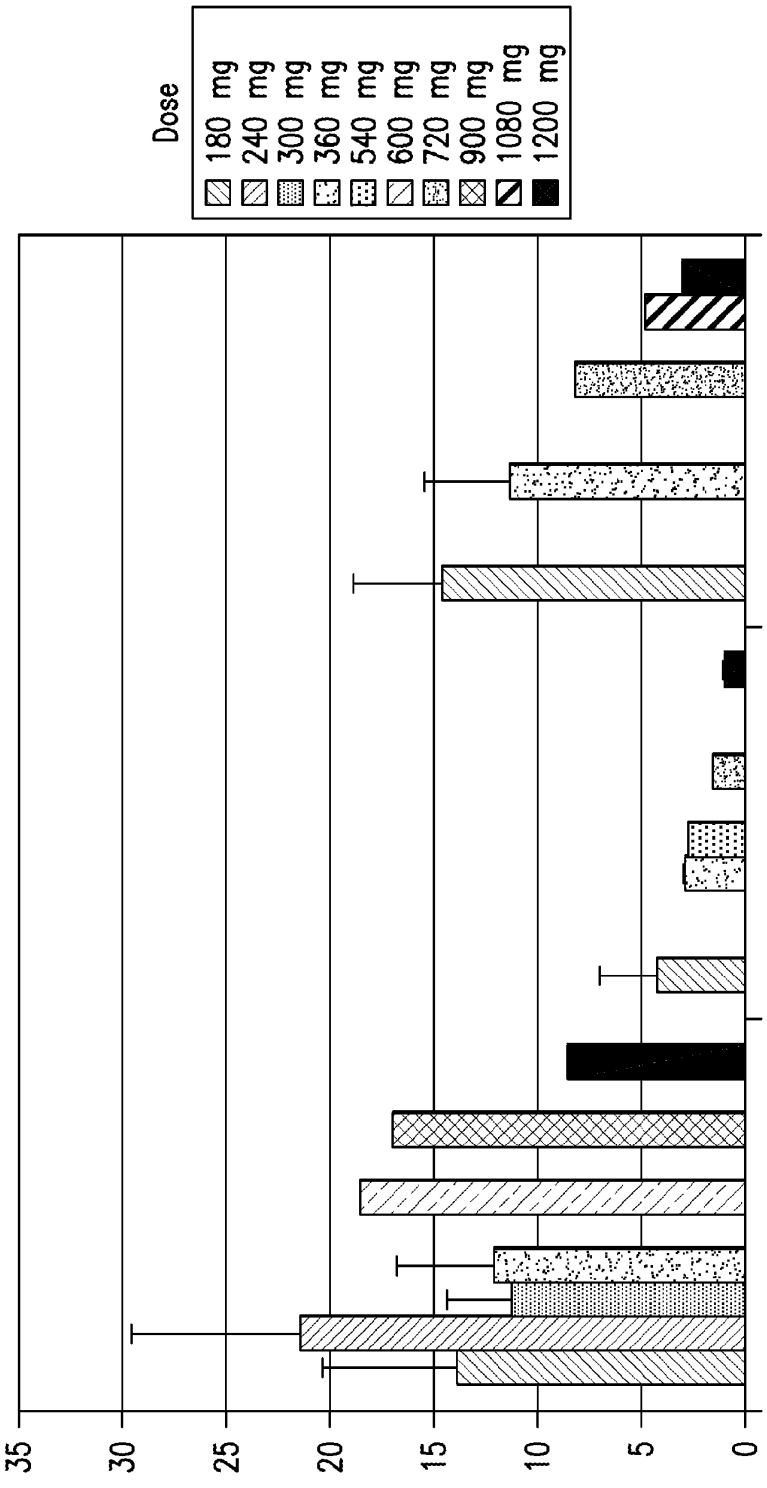


FIG.14

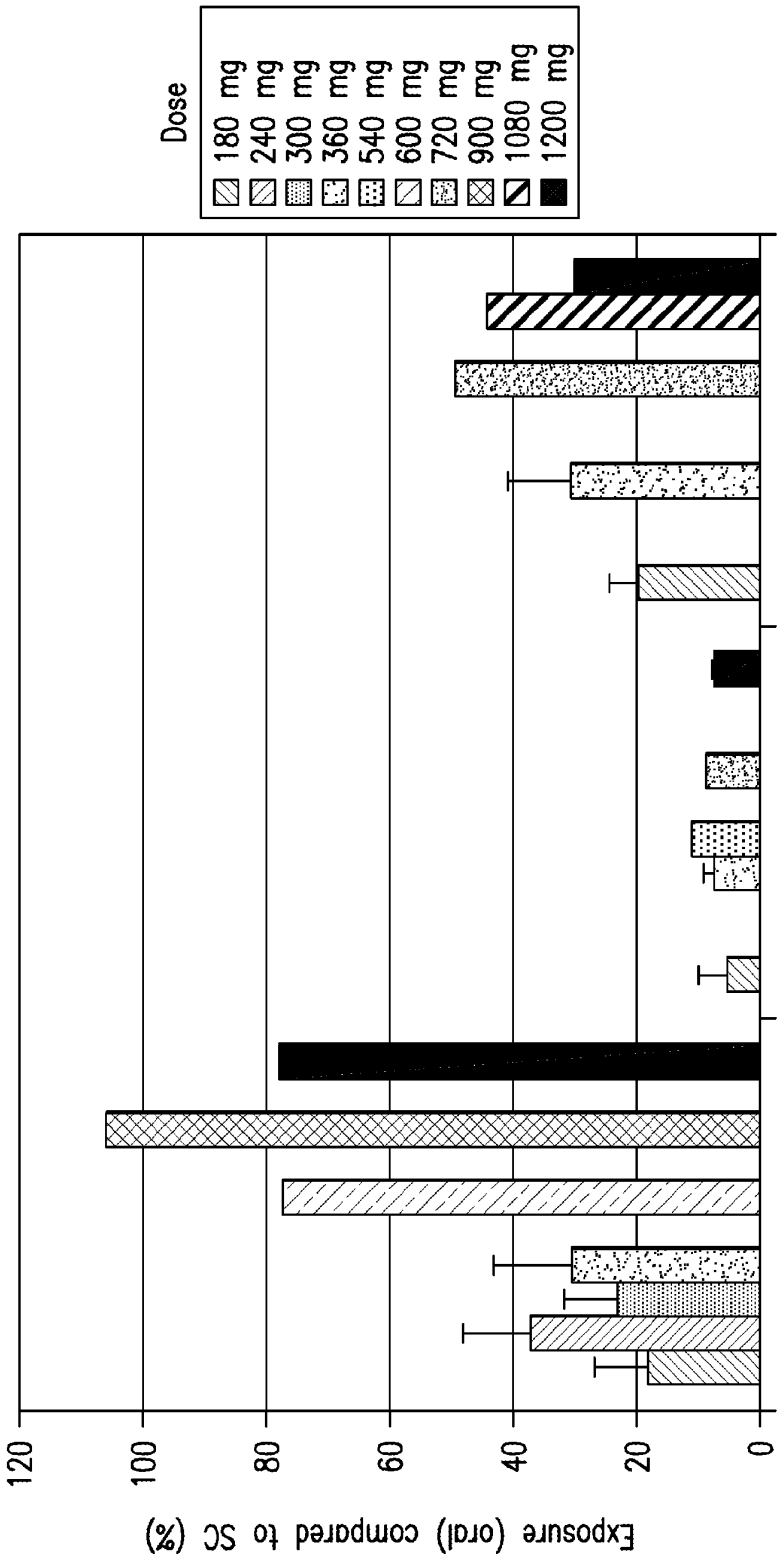


FIG.15

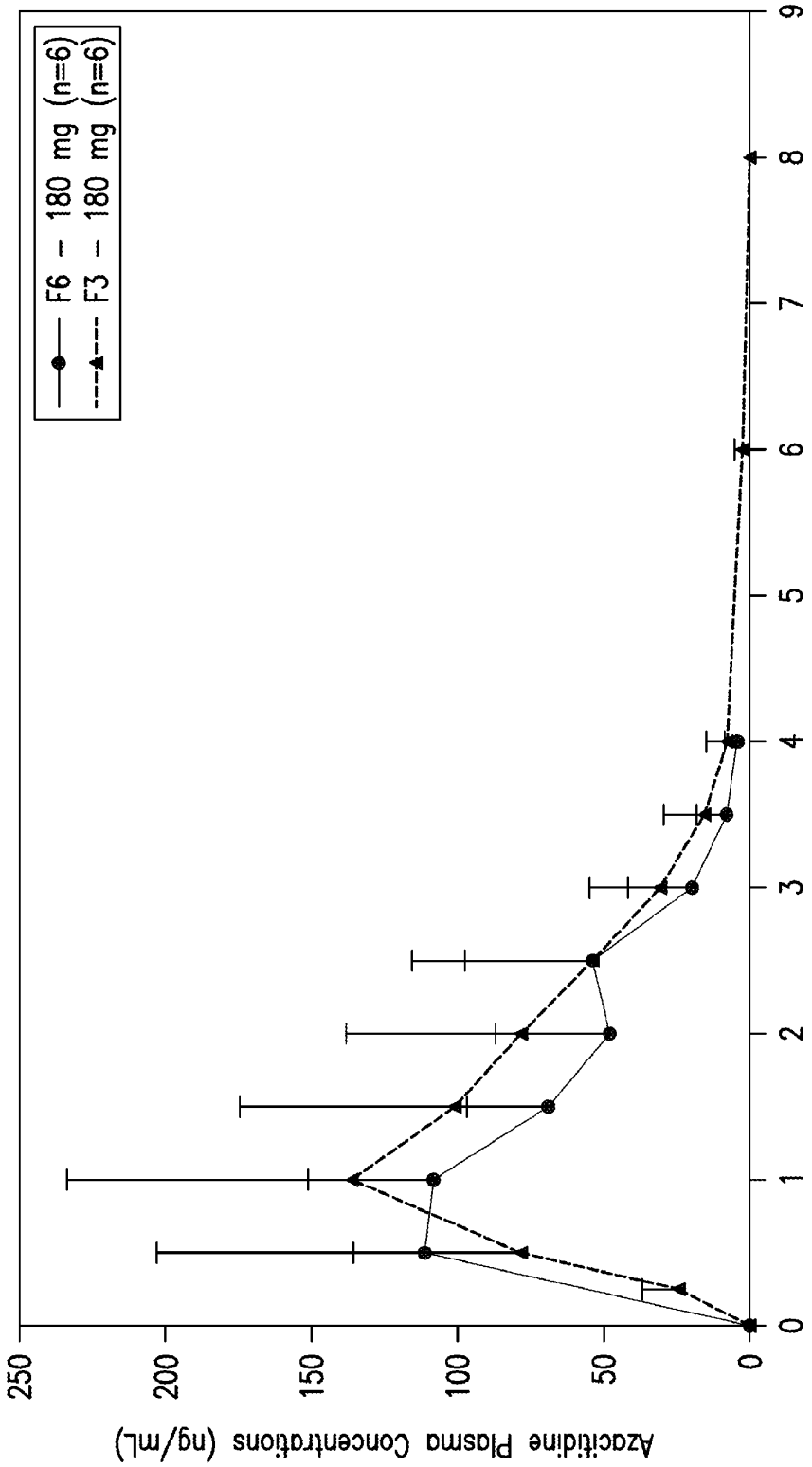


FIG. 16

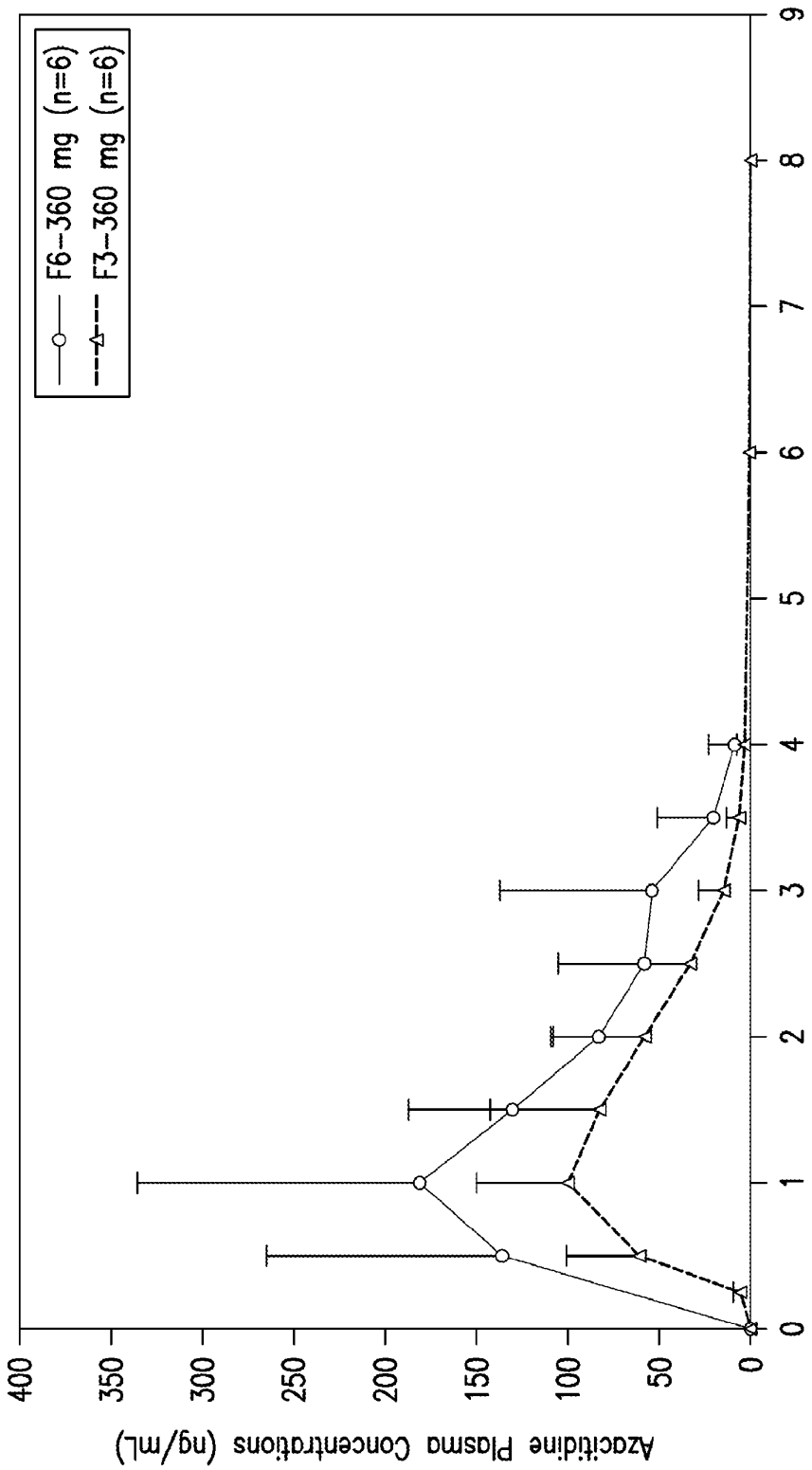


FIG.17

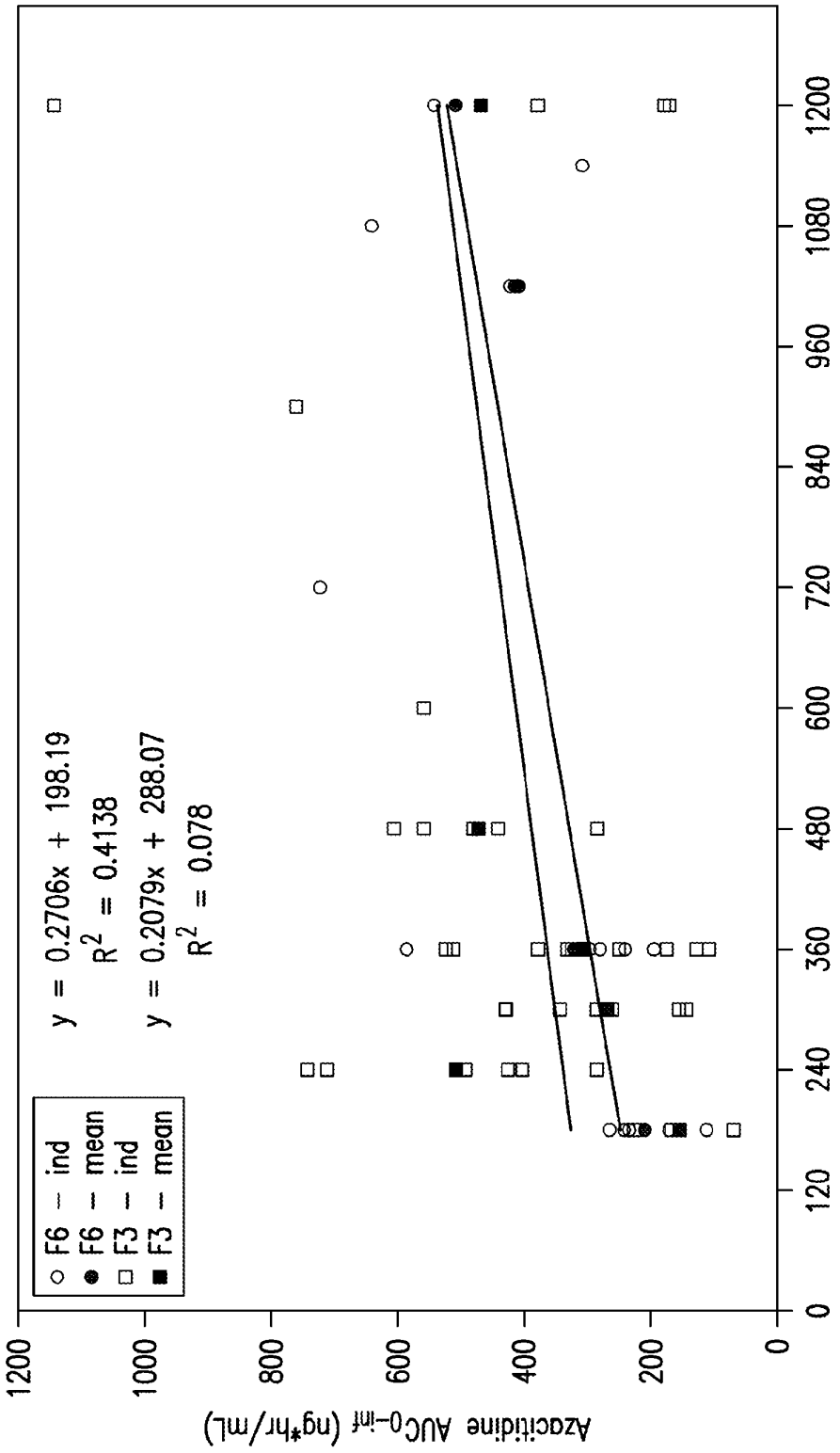


FIG. 18

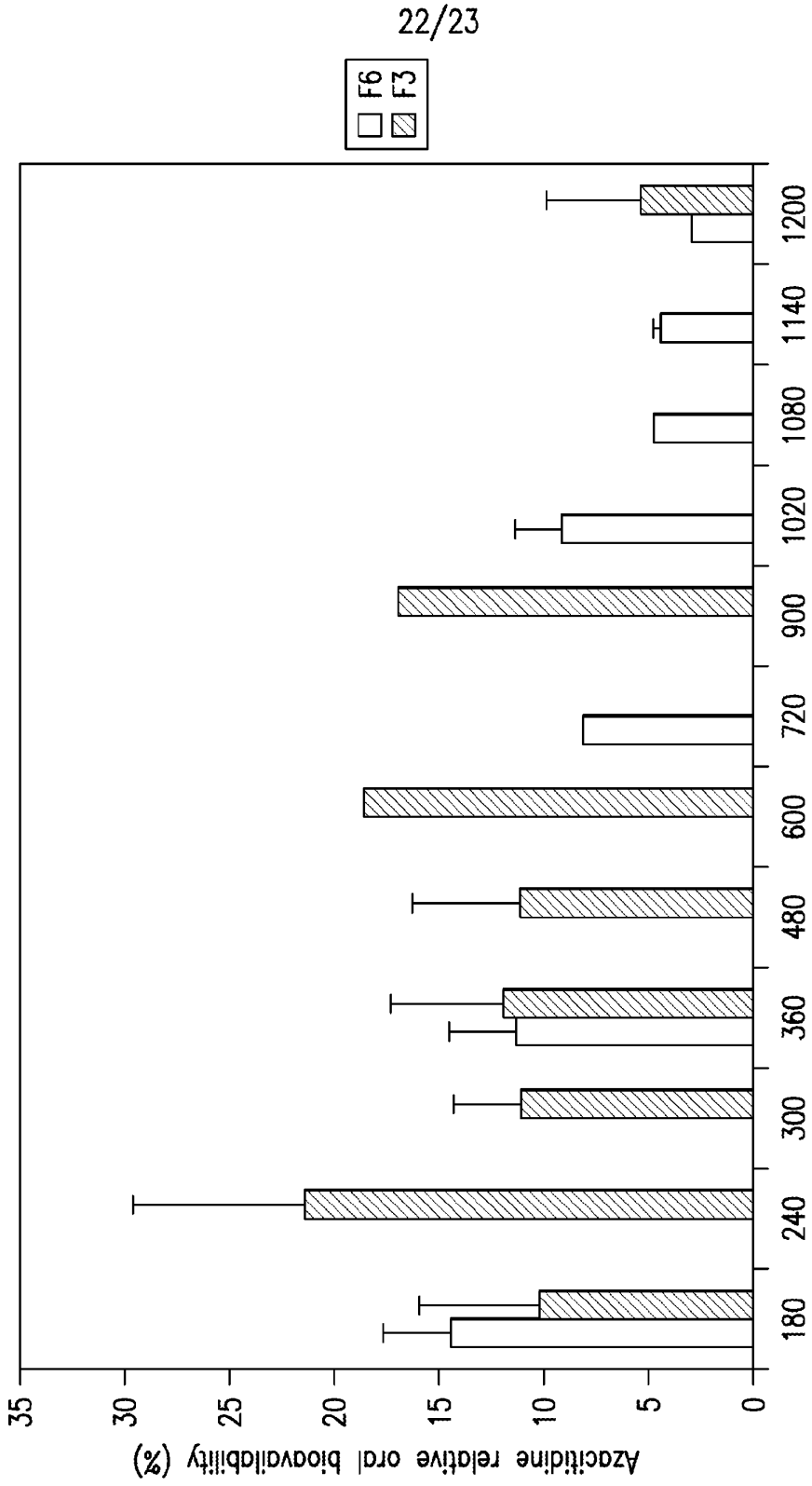


FIG.19

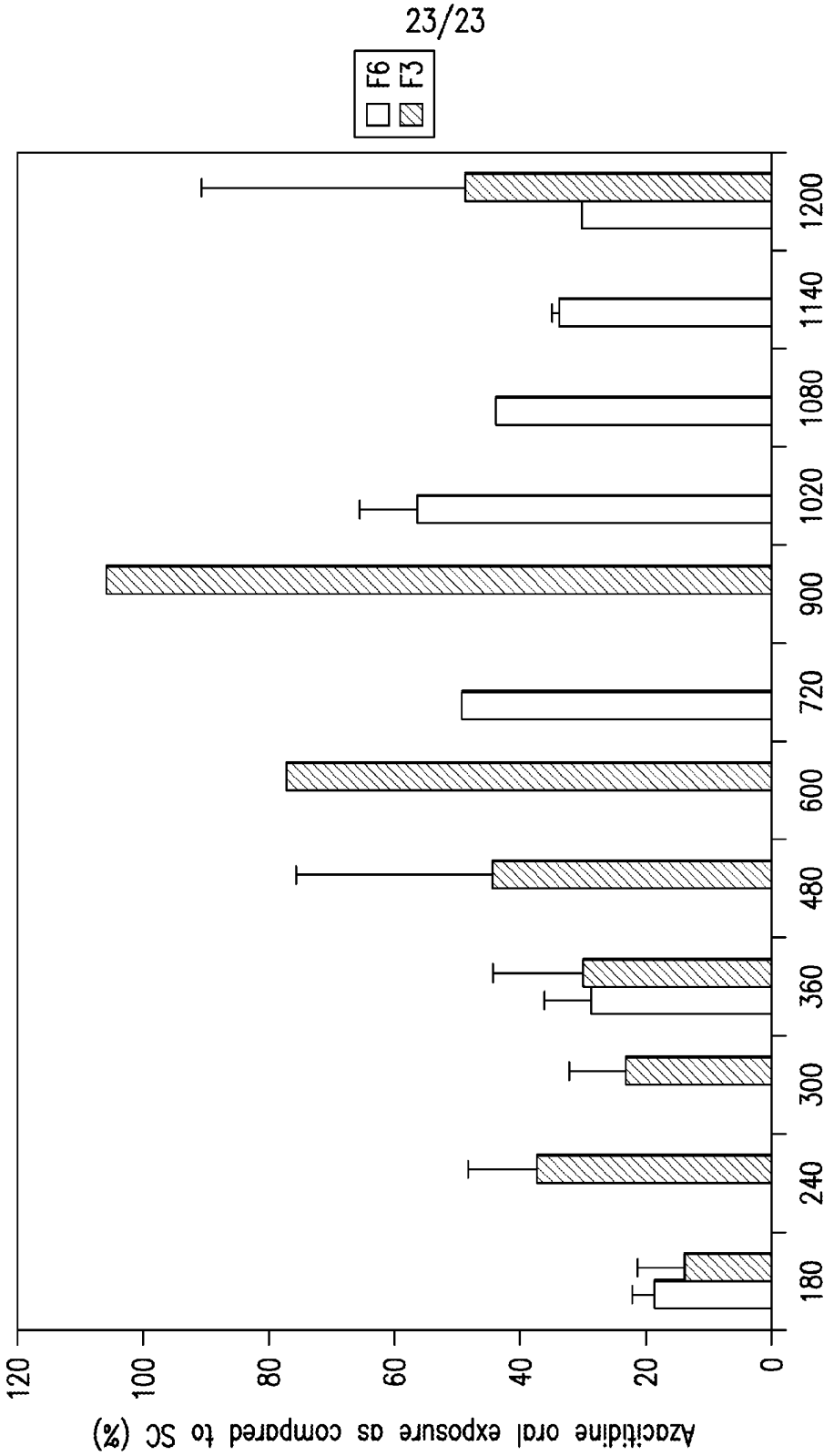


FIG.20

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2009/002999

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K9/20 A61K9/28 A61K31/7068

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, BIOSIS, FSTA, INSPEC

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2008/028193 A2 (PHARMION CORP [US]; ETTER JEFFREY B [US]) 6 March 2008 (2008-03-06) page 4, paragraph 20 examples 3-9 claims 1-42	1-74
X	US 2004/186065 A1 (IONESCU DUMITRU [US] ET AL) 23 September 2004 (2004-09-23) page 4, paragraph 46 - page 51, paragraph 56	1-6, 10-11, 16-34, 41, 45-50, 54-60, 62-72,74

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[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 September 2009	14/09/2009

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Schüle, Stefanie

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WARD: "An oral dosage formulation of azacitidine: A pilot pharmacokinetic study" JOURNAL OF CLINICAL ONCOLOGY, vol. 25, no. 18S, 1 July 2007 (2007-07-01) , XP009121612 the whole document	1-74
Y	STOLTZ: "Development of an Oral Dosage Form of Azacitidine: Overcoming Challenges in Chemistry, Formulation, and Bioavailability" ASH ANNUAL MEETING, vol. 108, 1 January 2006 (2006-01-01), XP009121611 the whole document	1-74

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2009/002999

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2008028193 A2	06-03-2008	US 2008057086 A1	06-03-2008
US 2004186065 A1	23-09-2004	EP 1610784 A2	04-01-2006
		US 2005137150 A1	23-06-2005
		WO 2004082619 A2	30-09-2004

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
27 May 2010 (27.05.2010)

PCT

(10) International Publication Number
WO 2010/059969 A2

(51) International Patent Classification:
A61K 39/395 (2006.01) *A61P 35/04* (2006.01)
A61K 31/00 (2006.01)

(21) International Application Number:
PCT/US2009/065381

(22) International Filing Date:
20 November 2009 (20.11.2009)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/117,102 22 November 2008 (22.11.2008) US
61/178,009 13 May 2009 (13.05.2009) US
61/179,307 18 May 2009 (18.05.2009) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2010/059969 A2

(54) Title: ANTI-ANGIOGENESIS THERAPY FOR THE TREATMENT OF BREAST CANCER

(57) Abstract: This invention concerns in general treatment of diseases and pathological conditions with anti-VEGF antibodies. More specifically, the invention concerns the treatment of human subjects susceptible to or diagnosed with breast cancer using an anti-VEGF antibody, preferably in combination with one or more additional anti-tumor therapeutic agents.

ANTI-ANGIOGENESIS THERAPY FOR THE TREATMENT OF BREAST CANCER

RELATED APPLICATIONS

This application claims priority to and the benefit of United States Provisional Application Serial No. 61/179,307, filed May 18, 2009, United States Provisional Application Serial No. 61/178,009, filed May 13, 2009, and United States Provisional Application Serial No. 61/117,102, filed November 22, 2008, the specifications of which are incorporated herein in their entirety.

FIELD OF THE INVENTION

This invention relates in general to treatment of human diseases and pathological conditions. More specifically, the invention relates to anti-angiogenesis therapy, either alone or in combination with other anti-cancer therapies, for the treatment of breast cancer.

BACKGROUND

Cancer remains to be one of the most deadly threats to human health. In the U.S., cancer affects nearly 1.3 million new patients each year, and is the second leading cause of death after heart disease, accounting for approximately 1 in 4 deaths. Breast cancer is the second most common form of cancer and the second leading cancer killer among American women. It is also predicted that cancer may surpass cardiovascular diseases as the number one cause of death within 5 years. Solid tumors are responsible for most of those deaths. Although there have been significant advances in the medical treatment of certain cancers, the overall 5-year survival rate for all cancers has improved only by about 10% in the past 20 years. Cancers, or malignant tumors, metastasize and grow rapidly in an uncontrolled manner, making timely detection and treatment extremely difficult.

Breast cancer is a disease that kills many women each year in the United States. According to the American Cancer Society, approximately 40,000 will die from the disease in 2008. Over 180,000 new cases of breast cancer are diagnosed annually, and it is estimated that one in eight women will develop breast cancer. These numbers indicate that breast cancer is one of the most dangerous diseases facing women today.

Metastatic breast cancer is generally incurable with only a few patients achieving long-term survival after standard chemotherapy. Greenberg et al., *J. Clin. Oncol.* 14:2197-2205 (1996).

Knowledge of the basic biology of breast cancer has expanded exponentially over the last three decades with some having an impact on therapy. A multinational, open-label phase II trial of 222 women with HER2 overexpressing metastatic breast cancer found a response rate of 15% with six confirmed complete responses using a recombinant humanized monoclonal antibody (trastuzumab, also known as Herceptin®, Genentech, South San Francisco) directed against HER2 (Cobleigh et al., *Proc. Am. Soc. Clin. Oncol.* 17:97 (1998)). A randomized phase III trial evaluated the safety and efficacy of adding Herceptin to first-line chemotherapy with either paclitaxel or the combination of doxorubicin plus cyclophosphamide. Overall response rate and time to progression significantly improved with the addition of Herceptin to chemotherapy compared to chemotherapy alone (Slamon et al., *Proc. Am. Soc. Clin. Oncol.* 17:98 (1998)). More importantly, the addition of Herceptin prolonged overall survival (Norton et al., *Proc. Am. Soc. Clin. Oncol.* 18:127a (1999)).

Though trastuzumab is the first novel, biologically-based therapeutic agent approved for the treatment of a subpopulation of breast cancer patients having HER2 overexpressing cancers, several other approaches have shown promise and have entered the clinic. There are estimates that 75 percent of women will newly diagnosed metastatic breast cancer are HER2-negative. Compounds which inhibit angiogenesis have generated particular interest for reaching additional breast cancer populations and have been and are the subject of clinical trials both in the US and abroad.

Angiogenesis is an important cellular event in which vascular endothelial cells proliferate, prune and reorganize to form new vessels from preexisting vascular network. There is compelling evidence that the development of a vascular supply is essential for normal and pathological proliferative processes (Folkman and Klagsbrun *Science* 235:442-447(1987)). Delivery of oxygen and nutrients, as well as the removal of catabolic products, represent rate-limiting steps in the majority of growth processes occurring in multicellular organisms.

While induction of new blood vessels is considered to be the predominant mode of tumor angiogenesis, recent data have indicated that some tumors may grow by co-opting existing host blood vessels. The co-opted vasculature then regresses, leading to tumor regression that is eventually reversed by hypoxia-induced angiogenesis at the tumor margin. Holash et al. *Science* 284:1994-1998 (1999).

One of the key positive regulators of both normal and abnormal angiogenesis is vascular endothelial growth factor (VEGF)-A. VEGF-A is part of a gene family including VEGF-B,

VEGF-C, VEGF-D, VEGF-E, VEGF-F, and PlGF. VEGF-A primarily binds to two high affinity receptor tyrosine kinases, VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR), the latter being the major transmitter of vascular endothelial cell mitogenic signals of VEGF-A. Additionally, neuropilin-1 has been identified as a receptor for heparin-binding VEGF-A isoforms, and may play a role in vascular development.

In addition to being an angiogenic factor in angiogenesis and vasculogenesis, VEGF, as a pleiotropic growth factor, exhibits multiple biological effects in other physiological processes, such as endothelial cell survival, vessel permeability and vasodilation, monocyte chemotaxis and calcium influx. Ferrara and Davis-Smyth (1997), *supra*. Moreover, studies have reported mitogenic effects of VEGF on a few non-endothelial cell types, such as retinal pigment epithelial cells, pancreatic duct cells and Schwann cells. Guerrin et al. *J. Cell Physiol.* 164:385-394 (1995); Oberg-Welsh et al. *Mol. Cell. Endocrinol.* 126:125-132 (1997); Sondell et al. *J. Neurosci.* 19:5731-5740 (1999).

The recognition of VEGF as a primary regulator of angiogenesis in pathological conditions has led to numerous attempts to block VEGF activities in conditions that involve pathological angiogenesis. VEGF expression is upregulated in a majority of malignancies and the overexpression of VEGF correlates with a more advanced stage or with a poorer prognosis in many solid tumors. Therefore, molecules that inhibit VEGF signaling pathways have been used for the treatment of relatively advanced solid tumors in which pathological angiogenesis is noted.

Since cancer is still one of the most deadly threats, additional cancer treatments for patients are needed. Specifically, treatments for patients with MBC are needed to improve control of disease to prevent symptoms, while minimizing toxicity. The invention addresses these and other needs, as will be apparent upon review of the following disclosure.

SUMMARY

The invention concerns uses of anti-VEGF antibody for effectively treating breast cancer patients for previously untreated metastic breast cancer. In particular, the invention provides data from a randomized phase III clinical trial of bevacizumab (AVASTIN®) in combination with chemotherapy regimes in subjects with previously untreated metastic breast cancer in human subjects. Such chemotherapy regimes include taxane therapy (e.g., docetaxel or paclitaxel protein-bound particles (e.g., Abraxane®)), anthracycline therapy (e.g., doxorubicin, epirubicin or combinations thereof) or capecitabine therapy. In some embodiments, the treatment is used as first line therapy for locally recurrent or previously

untreated metastatic breast cancer. The success of the trial shows that adding anti-VEGF antibody to a standard chemotherapy provides statistically significant and clinically meaningful benefits to breast cancer patients. In addition, safety was consistent with results of prior bevacizumab trials.

The results obtained in clinical studies of the use of bevacizumab in human subjects with metastatic breast cancer show that the efficacy, as evaluated by progression free survival (PFS) was positive especially when compared to PFS data for chemotherapeutic agents alone. Subjects in the clinical trials who received bevacizumab in combination with taxane therapy (e.g., docetaxel or paclitaxel protein-bound particles (e.g., Abraxane®))/anthracycline therapy (e.g., doxorubicin, epirubicin or combinations thereof) had an increase in progression free survival compared to subjects treated with the taxane therapy (e.g., docetaxel or paclitaxel protein-bound particles (e.g., Abraxane®))/anthracycline therapy (e.g., doxorubicin, epirubicin or combinations thereof) alone. Subjects in the clinical trials who received bevacizumab in combination with capecitabine therapy as described below, had an increase in progression free survival compared to subjects treated with capecitabine therapy alone. The difference was significantly significant.

Accordingly, provided herein are methods of treating a subject diagnosed with previously untreated metastatic breast cancer, comprising administering to the subject a treatment regimen comprising an effective amount of at least one chemotherapy and an anti-VEGF antibody, wherein said subject has not received any chemotherapy for locally recurrent or metastatic breast cancer. Optionally, the subject is HER2-negative. In some embodiments, the subject is HER2 positive. Optionally, the subject has not received prior adjuvant chemotherapy in recurrence less than or equal to 12 months since last dose. The treatment regimen combining the chemotherapy and the administration of the anti-VEGF effectively extends the progression free survival (PFS) of the subject. In certain embodiments, the treatment regimen combining the chemotherapy and the anti-VEGF antibody has a safety profile that is consistent with results of prior bevacizumab trials.

Further provided herein are uses of an anti-VEGF antibody with at least one chemotherapeutic agent in the manufacturer of a medicament for treating previously untreated metastatic breast cancer in a subject, wherein said subject has not received any chemotherapy for locally recurrent or metastatic breast cancer. Optionally, the subject is HER2-negative. In some embodiments, the subject is HER2 positive. Optionally, the subject has not received prior adjuvant chemotherapy in recurrence less than or equal to 12 months since last dose.

The use of the anti-VEGF and the chemotherapeutic agent effectively extends the progression free survival (PFS) of the subject. In certain embodiments, the use of the chemotherapy and the anti-VEGF antibody has a safety profile that is consistent with results of prior bevacizumab trials.

Provided also herein are anti-VEGF antibodies for use in a method of treating locally recurrent or metastatic breast cancer in a subject, the method comprising administering to the subject a treatment regimen comprising an effective amount of a chemotherapy and an anti-VEGF antibody, wherein said subject has not received any chemotherapy for locally recurrent or metastatic breast cancer. Optionally, the subject is HER2-negative. In some embodiments, the subject is HER2 positive. Optionally, the subject has not received prior adjuvant chemotherapy in recurrence less than or equal to 12 months since last dose. The treatment regimen combining the chemotherapy and the administration of the anti-VEGF effectively extends the progression free survival (PFS) of the subject. In certain embodiments, the treatment regimen combining the chemotherapy and the anti-VEGF antibody has a safety profile that is consistent with results of prior bevacizumab trials.

In certain embodiments of any of the methods, uses and compositions provided herein, the PFS is extended about 1 month, 1.2 months, 2 months, 2.4 months, 2.9 months, 3 months, 3.5 months, 4, months, 6 months, 7 months, 8 months, 9 months, 1 year, about 2 years, about 3 years, etc. In one embodiment, the PFS is extended about 2.9 months to 3.5 months (e.g., with capecitabine). In one embodiment, the PFS is extended about 1.2 months to about 2.4 months (e.g., with taxane/anthracycline).

Any chemotherapeutic agent exhibiting anticancer activity can be used according to any of the methods, uses and compositions provided herein. In certain embodiments, the chemotherapeutic agent is selected from the group consisting of alkylating agents, antimetabolites, folic acid analogs, pyrimidine analogs, purine analogs and related inhibitors, vinca alkaloids, epipodopyyllotoxins, antibiotics, L-Asparaginase, topoisomerase inhibitor, interferons, platinum cooridnation complexes, anthracenedione substituted urea, methyl hydrazine derivatives, adrenocortical suppressant, adrenocorticosteroides, progestins, estrogens, antiestrogen, androgens, antiandrogen, and gonadotropin-releasing hormone analog. In certain embodiments, the chemotherapeutic agent is for example, capecitabine, taxane, anthracycline, paclitaxel, docetaxel, paclitaxel protein-bound particles (e.g., Abraxane®), doxorubicin, epirubicin, 5-fluorouracil, cyclophosphamide or combinations

thereof. Two or more chemotherapeutic agents can be used (e.g., in a cocktail) to be administered in combination with administration of the anti-VEGF antibody.

Clinical benefits of the any of the methods, uses and compositions provided herein according to the invention can be measured by, for example, duration of progression free survival (PFS), time to treatment failure, objective response rate and duration of response.

Accordingly, the invention features a method of instructing a human subject with, e.g., breast, cancer by providing instructions to receive treatment with an anti-VEGF antibody so as to increase progression free survival of the subject, to decrease the subject's risk of cancer recurrence or to increase the subject's likelihood of survival. In some embodiments the method further comprises providing instructions to receive treatment with at least one chemotherapeutic agent. The treatment with the anti-VEGF antibody may be concurrent with or sequential to the treatment with the chemotherapeutic agent. In certain embodiments the subject is treated as instructed by the method of instructing.

The invention also provides a promotional method, comprising promoting the administration of an anti-VEGF antibody for treatment of, e.g., breast, cancer in a human subject. In some embodiments the method further comprises promoting the administration of at least one chemotherapeutic agent. Administration of the anti-VEGF antibody may be concurrent with or sequential to administration of the chemotherapeutic agent. Promotion may be conducted by any means available. In some embodiments the promotion is by a package insert accompanying a commercial formulation of the anti-VEGF antibody. The promotion may also be by a package insert accompanying a commercial formulation of the chemotherapeutic agent. Promotion may be by written or oral communication to a physician or health care provider. In some embodiments the promotion is by a package insert where the package inset provides instructions to receive therapy with anti-VEGF antibody. In some embodiments the promotion is followed by the treatment of the subject with the anti-VEGF antibody with or without the chemotherapeutic agent.

The invention provides a business method, comprising marketing an anti-VEGF antibody for treatment of, e.g., breast, cancer in a human subject so as to increase progression free survival, or decrease the subject's likelihood of cancer recurrence or increase the subject's likelihood of survival. In some embodiments the method further comprises marketing a chemotherapeutic agent for use in combination with the anti-VEGF antibody. In some embodiments the marketing is followed by treatment of the subject with the anti-VEGF antibody with or without the chemotherapeutic agent.

Also provided is a business method, comprising marketing a chemotherapeutic agent in combination with an anti-VEGF antibody for treatment of, e.g., breast, cancer in a human subject so as to increase progression free survival, or decrease the subject's likelihood of cancer recurrence or increase the subject's likelihood of survival. In some embodiments, the marketing is followed by treatment of the subject with the combination of the chemotherapeutic agent and the anti-VEGF antibody.

In any of the methods, uses and compositions provided herein, the anti-VEGF antibody may be substituted with a VEGF specific antagonist, e.g., a VEGF receptor molecule or chimeric VEGF receptor molecule as described herein. In certain embodiments of the methods, uses and compositions provided herein, the anti-VEGF antibody is bevacizumab. The anti-VEGF antibody, or antigen-binding fragment thereof, can be a monoclonal antibody, a chimeric antibody, a fully human antibody, or a humanized antibody. Exemplary antibodies useful in the methods of the invention include bevacizumab (AVASTIN®), a G6 antibody, a B20 antibody, and fragments thereof. In certain embodiments, the anti-VEGF antibody has a heavy chain variable region comprising the following amino acid sequence:

EVQLVESGGG LVQPGGSLRL SCAASGYTFT NYGMNWVRQA PGKGLEWVGW

INTYTGEPTY AADFKRRFTF SLDTSKSTAY LQMNSLRAED TAVYYCAKYP

HYYGSSHWYF DVWGQGTLVT VSS (SEQ ID No. 1)

and a light chain variable region comprising the following amino acid sequence:

DIQMTQSPSS LSASVGDRVT ITCSASQDIS NYLNWYQQKP GKAPKVLIYF

TSSLHSGVPS RFSGSGSGTD FTLTISSLQP EDFATYYCQQ YSTVPWTFGQ

GTKVEIKR (SEQ ID No. 2).

The antibody, or antigen-binding fragment thereof, can also be an antibody that lacks an Fc portion, an $F(ab')_2$, an Fab, or an Fv structure.

In one embodiment of the methods, uses and compositions provided herein, the treatment is a combination of a VEGF-specific antagonist, e.g., anti-VEGF antibody, and at least one chemotherapeutic agent. In other embodiments of the methods, uses and compositions provided herein, the VEGF-specific antagonist is a monotherapy.

Each of any of the methods, uses and compositions provided herein may be practiced in relation to the treatment of cancers including, but not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include breast

cancer, squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, colon cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, renal cancer, vulval cancer, thyroid cancer, hepatic carcinoma, gastric cancer, melanoma, and various types of head and neck cancer. In some embodiments of the methods of the invention the subject has metastatic breast cancer. In some embodiments of the methods, uses and compositions provided herein the subject has previously untreated metastatic breast cancer. In some embodiments the subject has HER2-negative metastic breast cancer.

Each of the above aspects can further include monitoring the subject for recurrence of the cancer. Monitoring can be accomplished, for example, by evaluating progression free survival (PFS) or overall survival (OS) or objective response rate (ORR). In one embodiment, the PFS or the OS or the ORR is evaluated after initiation of treatment.

Depending on the type and severity of the disease, preferred dosages for the anti-VEGF antibody, e.g., bevacizumab, are described herein and can range from about 1μg/kg to about 50 mg/kg, most preferably from about 5 mg/kg to about 15 mg/kg, including but not limited to 5 mg/kg, 7.5 mg/kg, 10 mg/kg or 15 mg/kg. The frequency of administration will vary depending on the type and severity of the disease. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until the cancer is treated or the desired therapeutic effect is achieved, as measured by the methods described herein or known in the art. In one example, the anti-VEGF antibody is administered once every week, every two weeks, or every three weeks, at a dose range from about 5 mg/kg to about 15 mg/kg, including but not limited to 5 mg/kg, 7.5 mg/kg, 10 mg/kg or 15 mg/kg. However, other dosage regimens may be useful. The progress of the therapy of the invention is easily monitored by conventional techniques and assays.

In additional embodiments of each of the above aspects, the VEGF-specific antagonist, e.g., anti-VEGF antibody is administered locally or systemically (e.g., orally or intravenously). In other embodiments, one aspect of the treatment is with the VEGF-specific antagonist in a monotherapy or a monotherapy for the duration of the VEGF-specific antagonist treatment period, e.g., in extended treatment phase or maintenance therapy, as assessed by the clinician or described herein.

In other embodiments of the methods, uses and compositions provided herein, treatment, use or composition with the VEGF-specific antagonist is in combination with an additional anti-cancer therapy, including but not limited to, surgery, radiation therapy, chemotherapy, differentiating therapy, biotherapy, immune therapy, an angiogenesis inhibitor, a cytotoxic agent and/or an anti-proliferative compound. Treatment, use and composition with the VEGF-specific antagonist can also include any combination of the above types of therapeutic regimens. In some embodiments, the chemotherapeutic agent and the VEGF-specific antagonist are administered concurrently.

In the embodiments of the methods, uses and compositions provided herein which include an additional anti-cancer therapy, the subject can be further treated with the additional anti-cancer therapy before, during (e.g., simultaneously), or after administration of the VEGF-specific antagonist. In one embodiment, the VEGF-specific antagonist, administered either alone or with an anti-cancer therapy, can be administered as maintenance therapy.

Other features and advantages of the invention will be apparent from the following Detailed Description, the drawings, and the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the study design for the metastatic breast cancer trial using bevacizumab (BV) or placebo (PL) with various chemotherapies.

Figure 2 depicts progression free survival (PFS) curves for capecitabine arm of the trial. INV (investigator) is PFS assessed by investigator and IRC is PFS assessed by independent review committee (IRC), where placebo is PL and bevacizumab is BV.

Figure 3 depicts PFS curves for taxane/anthracycline arm of the trial. INV is PFS assessed by investigator and IRC is PFS assessed by independent review committee (IRC), where placebo is PL and bevacizumab is BV.

Figure 4 depicts a subgroup analyses of PFS in the capecitabine and taxane/anthracycline groups of the trial.

Figure 5 depicts the objective response rate for capecitabine (Cape) and taxane/anthracycline (T/Antra) groups.

Figure 6 depicts a subgroup analysis of PFS for taxane/anthracycline (T/Anthra) cohorts.

DETAILED DESCRIPTION

I. DEFINITIONS

The term "VEGF" or "VEGF-A" is used to refer to the 165-amino acid human vascular endothelial cell growth factor and related 121-, 145-, 189-, and 206- amino acid human vascular endothelial cell growth factors, as described by, e.g., Leung et al. *Science*, 246:1306 (1989), and Houck et al. *Mol. Endocrin.*, 5:1806 (1991), together with the naturally occurring allelic and processed forms thereof. VEGF-A is part of a gene family including VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F, and PlGF. VEGF-A primarily binds to two high affinity receptor tyrosine kinases, VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR), the latter being the major transmitter of vascular endothelial cell mitogenic signals of VEGF-A. Additionally, neuropilin-1 has been identified as a receptor for heparin-binding VEGF-A isoforms, and may play a role in vascular development. The term "VEGF" or "VEGF-A" also refers to VEGFs from non-human species such as mouse, rat, or primate. Sometimes the VEGF from a specific species is indicated by terms such as hVEGF for human VEGF or mVEGF for murine VEGF. Typically, VEGF refers to human VEGF. The term "VEGF" is also used to refer to truncated forms or fragments of the polypeptide comprising amino acids 8 to 109 or 1 to 109 of the 165-amino acid human vascular endothelial cell growth factor. Reference to any such forms of VEGF may be identified in the application, e.g., by "VEGF (8-109)," "VEGF (1-109)" or "VEGF165." The amino acid positions for a "truncated" native VEGF are numbered as indicated in the native VEGF sequence. For example, amino acid position 17 (methionine) in truncated native VEGF is also position 17 (methionine) in native VEGF. The truncated native VEGF has binding affinity for the KDR and Flt-1 receptors comparable to native VEGF.

An "anti-VEGF antibody" is an antibody that binds to VEGF with sufficient affinity and specificity. The antibody selected will normally have a binding affinity for VEGF, for example, the antibody may bind hVEGF with a Kd value of between 100 nM-1 pM. Antibody affinities may be determined by a surface plasmon resonance based assay (such as the BIAcore assay as described in PCT Application Publication No. WO2005/012359); enzyme-linked immunoabsorbent assay (ELISA); and competition assays (e.g. RIA's), for example. In certain embodiments, the anti-VEGF antibody of the invention can be used as a therapeutic agent in targeting and interfering with diseases or conditions wherein the VEGF activity is involved. Also, the antibody may be subjected to other biological activity assays, e.g., in order to evaluate its effectiveness as a therapeutic. Such assays are known in the art and

depend on the target antigen and intended use for the antibody. Examples include the HUVEC inhibition assay; tumor cell growth inhibition assays (as described in WO 89/06692, for example); antibody-dependent cellular cytotoxicity (ADCC) and complement-mediated cytotoxicity (CDC) assays (US Patent 5,500,362); and agonistic activity or hematopoiesis assays (see WO 95/27062). An anti-VEGF antibody will usually not bind to other VEGF homologues such as VEGF-B or VEGF-C, nor other growth factors such as PlGF, PDGF or bFGF.

A "VEGF antagonist" refers to a molecule capable of neutralizing, blocking, inhibiting, abrogating, reducing or interfering with VEGF activities including its binding to one or more VEGF receptors. VEGF antagonists include anti-VEGF antibodies and antigen-binding fragments thereof, receptor molecules and derivatives which bind specifically to VEGF thereby sequestering its binding to one or more receptors, anti-VEGF receptor antibodies and VEGF receptor antagonists such as small molecule inhibitors of the VEGFR tyrosine kinases.

A "native sequence" polypeptide comprises a polypeptide having the same amino acid sequence as a polypeptide derived from nature. Thus, a native sequence polypeptide can have the amino acid sequence of naturally-occurring polypeptide from any mammal. Such native sequence polypeptide can be isolated from nature or can be produced by recombinant or synthetic means. The term "native sequence" polypeptide specifically encompasses naturally-occurring truncated or secreted forms of the polypeptide (*e.g.*, an extracellular domain sequence), naturally-occurring variant forms (*e.g.,* alternatively spliced forms) and naturally-occurring allelic variants of the polypeptide.

A polypeptide "variant" means a biologically active polypeptide having at least about 80% amino acid sequence identity with the native sequence polypeptide. Such variants include, for instance, polypeptides wherein one or more amino acid residues are added, or deleted, at the N- or C-terminus of the polypeptide. Ordinarily, a variant will have at least about 80% amino acid sequence identity, more preferably at least about 90% amino acid sequence identity, and even more preferably at least about 95% amino acid sequence identity with the native sequence polypeptide.

The term "antibody" is used in the broadest sense and includes monoclonal antibodies (including full length or intact monoclonal antibodies), polyclonal antibodies, multivalent antibodies, multispecific antibodies (*e.g.,* bispecific antibodies), and antibody fragments (see below) so long as they exhibit the desired biological activity.

Throughout the present specification and claims, the numbering of the residues in an immunoglobulin heavy chain is that of the EU index as in Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991), expressly incorporated herein by reference. The "EU index as in Kabat" refers to the residue numbering of the human IgG1 EU antibody.

The "Kd" or "Kd value" according to this invention is in one embodiment measured by a radiolabeled VEGF binding assay (RIA) performed with the Fab version of the antibody and a VEGF molecule as described by the following assay that measures solution binding affinity of Fabs for VEGF by equilibrating Fab with a minimal concentration of (^{125}I)-labeled VEGF(109) in the presence of a titration series of unlabeled VEGF, then capturing bound VEGF with an anti-Fab antibody-coated plate (Chen, et al., (1999) *J. Mol Biol* 293:865-881). In one example, to establish conditions for the assay, microtiter plates (Dynex) are coated overnight with 5 ug/ml of a capturing anti-Fab antibody (Cappel Labs) in 50 mM sodium carbonate (pH 9.6), and subsequently blocked with 2% (w/v) bovine serum albumin in PBS for two to five hours at room temperature (approximately 23°C). In a non-adsorbant plate (Nunc #269620), 100 pM or 26 pM [^{125}I]VEGF(109) are mixed with serial dilutions of a Fab of interest, e.g., Fab-12 (Presta et al., (1997) *Cancer Res.* 57:4593-4599). The Fab of interest is then incubated overnight; however, the incubation may continue for 65 hours to insure that equilibrium is reached. Thereafter, the mixtures are transferred to the capture plate for incubation at room temperature for one hour. The solution is then removed and the plate washed eight times with 0.1% Tween-20 in PBS. When the plates had dried, 150 ul/well of scintillant (MicroScint-20; Packard) is added, and the plates are counted on a Topcount gamma counter (Packard) for ten minutes. Concentrations of each Fab that give less than or equal to 20% of maximal binding are chosen for use in competitive binding assays.

According to another embodiment the Kd or Kd value is measured by using surface plasmon resonance assays using a BIAcore™-2000 or a BIAcore™-3000 (BIAcore, Inc., Piscataway, NJ) at 25°C with immobilized hVEGF (8-109) CM5 chips at ~10 response units (RU). Briefly, carboxymethylated dextran biosensor chips (CM5, BIAcore Inc.) are activated with *N*-ethyl-*N'*- (3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) according to the supplier's instructions. Human VEGF is diluted with 10mM sodium acetate, pH 4.8, into 5ug/ml (~0.2uM) before injection at a flow rate of 5ul/minute to achieve approximately 10 response units (RU) of coupled protein. Following the injection of human VEGF, 1M ethanolamine is injected to block unreacted groups. For kinetics measurements, two-fold serial dilutions of Fab (0.78 nM to 500 nM) are injected in

PBS with 0.05% Tween 20 (PBST) at 25°C at a flow rate of approximately 25ul/min. Association rates (k_{on}) and dissociation rates (k_{off}) are calculated using a simple one-to-one Langmuir binding model (BIAcore Evaluation Software version 3.2) by simultaneous fitting the association and dissociation sensorgram. The equilibrium dissociation constant (Kd) was calculated as the ratio k_{off}/k_{on}. See, e.g., Chen, Y., et al., (1999) *J. Mol Biol* 293:865-881. If the on-rate exceeds 10^6 M^{-1} S^{-1} by the surface plasmon resonance assay above, then the on-rate is can be determined by using a fluorescent quenching technique that measures the increase or decrease in fluorescence emission intensity (excitation = 295 nm; emission = 340 nm, 16 nm band-pass) at 25°C of a 20nM anti-VEGF antibody (Fab form) in PBS, pH 7.2, in the presence of increasing concentrations of human VEGF short form (8-109) or mouse VEGF as measured in a spectrometer, such as a stop-flow equipped spectrophometer (Aviv Instruments) or a 8000-series SLM-Aminco spectrophotometer (ThermoSpectronic) with a stirred cuvette.

A "blocking" antibody or an antibody "antagonist" is one which inhibits or reduces biological activity of the antigen it binds. For example, a VEGF-specific antagonist antibody binds VEGF and inhibits the ability of VEGF to induce angiogenesis, to induce vascular endothelial cell proliferation or to induce vascular permeability. In certain embodiments, blocking antibodies or antagonist antibodies completely inhibit the biological activity of the antigen.

Unless indicated otherwise, the expression "multivalent antibody" is used throughout this specification to denote an antibody comprising three or more antigen binding sites. For example, the multivalent antibody is engineered to have the three or more antigen binding sites and is generally not a native sequence IgM or IgA antibody.

"Antibody fragments" comprise only a portion of an intact antibody, generally including an antigen binding site of the intact antibody and thus retaining the ability to bind antigen. Examples of antibody fragments encompassed by the present definition include: (i) the Fab fragment, having VL, CL, VH and CH1 domains; (ii) the Fab' fragment, which is a Fab fragment having one or more cysteine residues at the C-terminus of the CH1 domain; (iii) the Fd fragment having VH and CH1 domains; (iv) the Fd' fragment having VH and CH1 domains and one or more cysteine residues at the C-terminus of the CH1 domain; (v) the Fv fragment having the VL and VH domains of a single arm of an antibody; (vi) the dAb fragment (Ward *et al., Nature* 341, 544-546 (1989)) which consists of a VH domain; (vii) isolated CDR regions; (viii) $F(ab')_2$ fragments, a bivalent fragment including two Fab' fragments linked by a disulphide bridge at the hinge region; (ix) single chain antibody molecules (*e.g.* single chain Fv; scFv) (Bird *et al.*, *Science* 242:423-426 (1988); and Huston *et*

al., PNAS (USA) 85:5879-5883 (1988)); (x) "diabodies" with two antigen binding sites, comprising a heavy chain variable domain (VH) connected to a light chain variable domain (VL) in the same polypeptide chain (see, *e.g.*, EP 404,097; WO 93/11161; and Hollinger *et al., Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993)); (xi) "linear antibodies" comprising a pair of tandem Fd segments (VH-CH1-VH-CH1) which, together with complementary light chain polypeptides, form a pair of antigen binding regions (Zapata *et al. Protein Eng.* 8(10):1057-1062 (1995); and US Patent No. 5,641,870).

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigen. Furthermore, in contrast to polyclonal antibody preparations that typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. The modifier "monoclonal" is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the invention may be made by the hybridoma method first described by Kohler *et al., Nature* 256:495 (1975), or may be made by recombinant DNA methods (see, *e.g.*, U.S. Patent No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson *et al., Nature* 352:624-628 (1991) or Marks *et al., J. Mol. Biol.* 222:581-597 (1991), for example.

An "Fv" fragment is an antibody fragment which contains a complete antigen recognition and binding site. This region consists of a dimer of one heavy and one light chain variable domain in tight association, which can be covalent in nature, for example in scFv. It is in this configuration that the three CDRs of each variable domain interact to define an antigen binding site on the surface of the V_H-V_L dimer. Collectively, the six CDRs or a subset thereof confer antigen binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although usually at a lower affinity than the entire binding site.

As used herein, "antibody variable domain" refers to the portions of the light and heavy chains of antibody molecules that include amino acid sequences of Complementarity Determining Regions (CDRs; ie., CDR1, CDR2, and CDR3), and Framework Regions (FRs). V_H refers to the variable domain of the heavy chain. V_L refers to the variable domain of the

light chain. According to the methods used in this invention, the amino acid positions assigned to CDRs and FRs may be defined according to Kabat (Sequences of Proteins of Immunological Interest (National Institutes of Health, Bethesda, Md., 1987 and 1991)). Amino acid numbering of antibodies or antigen binding fragments is also according to that of Kabat.

As used herein, the term "Complementarity Determining Regions" (CDRs; i.e., CDR1, CDR2, and CDR3) refers to the amino acid residues of an antibody variable domain the presence of which are necessary for antigen binding. Each variable domain typically has three CDR regions identified as CDR1, CDR2 and CDR3. Each complementarity determining region may comprise amino acid residues from a "complementarity determining region" as defined by Kabat (*i.e.* about residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy chain variable domain; Kabat *et al., Sequences of Proteins of Immunological Interest,* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)) and/or those residues from a "hypervariable loop" (*i.e.* about residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the light chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy chain variable domain; Chothia and Lesk *J. Mol. Biol.* 196:901-917 (1987)). In some instances, a complementarity determining region can include amino acids from both a CDR region defined according to Kabat and a hypervariable loop. For example, the CDRH1 of the heavy chain of antibody 4D5 includes amino acids 26 to 35.

"Framework regions" (hereinafter FR) are those variable domain residues other than the CDR residues. Each variable domain typically has four FRs identified as FR1, FR2, FR3 and FR4. If the CDRs are defined according to Kabat, the light chain FR residues are positioned at about residues 1-23 (LCFR1), 35-49 (LCFR2), 57-88 (LCFR3), and 98-107 (LCFR4) and the heavy chain FR residues are positioned about at residues 1-30 (HCFR1), 36-49 (HCFR2), 66-94 (HCFR3), and 103-113 (HCFR4) in the heavy chain residues. If the CDRs comprise amino acid residues from hypervariable loops, the light chain FR residues are positioned about at residues 1-25 (LCFR1), 33-49 (LCFR2), 53-90 (LCFR3), and 97-107 (LCFR4) in the light chain and the heavy chain FR residues are positioned about at residues 1-25 (HCFR1), 33-52 (HCFR2), 56-95 (HCFR3), and 102-113 (HCFR4) in the heavy chain residues. In some instances, when the CDR comprises amino acids from both a CDR as defined by Kabat and those of a hypervariable loop, the FR residues will be adjusted accordingly. For example, when CDRH1 includes amino acids H26-H35, the heavy chain FR1 residues are at positions 1-25 and the FR2 residues are at positions 36-49.

The "Fab" fragment contains a variable and constant domain of the light chain and a variable domain and the first constant domain (CH1) of the heavy chain. $F(ab')_2$ antibody fragments comprise a pair of Fab fragments which are generally covalently linked near their carboxy termini by hinge cysteines between them. Other chemical couplings of antibody fragments are also known in the art.

"Single-chain Fv" or "scFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Generally the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains, which enables the scFv to form the desired structure for antigen binding. For a review of scFv, see Pluckthun in *The Pharmacology of Monoclonal Antibodies,* Vol 113, Rosenburg and Moore eds. Springer-Verlag, New York, pp. 269-315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy chain variable domain (V_H) connected to a light chain variable domain (V_L) in the same polypeptide chain (V_H and V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger et al., *Proc. Natl. Acad. Sci. USA,* 90:6444-6448 (1993).

The expression "linear antibodies" refers to the antibodies described in Zapata et al., *Protein Eng.,* 8(10):1057-1062 (1995). Briefly, these antibodies comprise a pair of tandem Fd segments (V_H-C_H1-V_H-C_H1) which, together with complementary light chain polypeptides, form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Patent No. 4,816,567; and Morrison et al., *Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984)).

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric antibodies which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a

hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones *et al., Nature* 321:522-525 (1986); Riechmann et al., *Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992).

A "human antibody" is one which possesses an amino acid sequence which corresponds to that of an antibody produced by a human and/or has been made using any of the techniques for making human antibodies as disclosed herein. This definition of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues. Human antibodies can be produced using various techniques known in the art. In one embodiment, the human antibody is selected from a phage library, where that phage library expresses human antibodies (Vaughan et al. *Nature Biotechnology* 14:309-314 (1996): Sheets et al. *Proc. Natl. Acad. Sci.* 95:6157-6162 (1998)); Hoogenboom and Winter, *J. Mol. Biol.,* 227:381 (1991); Marks et al., *J. Mol. Biol.*, 222:581 (1991)). Human antibodies can also be made by introducing human immunoglobulin loci into transgenic animals, e.g., mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. Upon challenge, human antibody production is observed, which closely resembles that seen in humans in all respects, including gene rearrangement, assembly, and antibody repertoire. This approach is described, for example, in U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, and in the following scientific publications: Marks et al., *Bio/Technology* 10: 779-783 (1992); Lonberg et al., *Nature* 368: 856-859 (1994); Morrison, *Nature* 368:812-13 (1994); Fishwild et al., *Nature Biotechnology* 14: 845-51 (1996); Neuberger, *Nature Biotechnology* 14: 826 (1996); Lonberg and Huszar, *Intern. Rev. Immunol.* 13:65-93 (1995). Alternatively, the human antibody may be prepared via immortalization of human B lymphocytes producing an antibody directed against a target antigen (such B lymphocytes may be recovered from an individual or may have been immunized in vitro).

See, e.g., Cole et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985); Boerner et al., *J. Immunol.,* 147 (1):86-95 (1991); and U.S. Pat. No. 5,750,373.

An "affinity matured" antibody is one with one or more alterations in one or more CDRs thereof which result an improvement in the affinity of the antibody for antigen, compared to a parent antibody which does not possess those alteration(s). Preferred affinity matured antibodies will have nanomolar or even picomolar affinities for the target antigen. Affinity matured antibodies are produced by procedures known in the art. Marks et al. Bio/Technology 10:779-783 (1992) describes affinity maturation by VH and VL domain shuffling. Random mutagenesis of CDR and/or framework residues is described by: Barbas et al. *Proc Nat. Acad. Sci, USA* 91:3809-3813 (1994); Schier et al. *Gene* 169:147-155 (1995); Yelton et al. *J. Immunol.* 155:1994-2004 (1995); Jackson et al., *J. Immunol.* 154(7):3310-9 (1995); and Hawkins et al., *J. Mol. Biol.* 226:889-896 (1992).

A "functional antigen binding site" of an antibody is one which is capable of binding a target antigen. The antigen binding affinity of the antigen binding site is not necessarily as strong as the parent antibody from which the antigen binding site is derived, but the ability to bind antigen must be measurable using any one of a variety of methods known for evaluating antibody binding to an antigen. Moreover, the antigen binding affinity of each of the antigen binding sites of a multivalent antibody herein need not be quantitatively the same. For the multimeric antibodies herein, the number of functional antigen binding sites can be evaluated using ultracentrifugation analysis as described in Example 2 of U.S. Patent Application Publication No. 20050186208. According to this method of analysis, different ratios of target antigen to multimeric antibody are combined and the average molecular weight of the complexes is calculated assuming differing numbers of functional binding sites. These theoretical values are compared to the actual experimental values obtained in order to evaluate the number of functional binding sites.

An antibody having a "biological characteristic" of a designated antibody is one which possesses one or more of the biological characteristics of that antibody which distinguish it from other antibodies that bind to the same antigen.

In order to screen for antibodies which bind to an epitope on an antigen bound by an antibody of interest, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed.

A "species-dependent antibody" is one which has a stronger binding affinity for an antigen from a first mammalian species than it has for a homologue of that antigen from a

second mammalian species. Normally, the species-dependent antibody "binds specifically" to a human antigen (*i.e.* has a binding affinity (K_d) value of no more than about 1 x 10^{-7} M, preferably no more than about 1 x 10^{-8} M and most preferably no more than about 1 x 10^{-9} M) but has a binding affinity for a homologue of the antigen from a second nonhuman mammalian species which is at least about 50 fold, or at least about 500 fold, or at least about 1000 fold, weaker than its binding affinity for the human antigen. The species-dependent antibody can be any of the various types of antibodies as defined above, but typically is a humanized or human antibody.

As used herein, "antibody mutant" or "antibody variant" refers to an amino acid sequence variant of the species-dependent antibody wherein one or more of the amino acid residues of the species-dependent antibody have been modified. Such mutants necessarily have less than 100% sequence identity or similarity with the species-dependent antibody. In one embodiment, the antibody mutant will have an amino acid sequence having at least 75% amino acid sequence identity or similarity with the amino acid sequence of either the heavy or light chain variable domain of the species-dependent antibody, more preferably at least 80%, more preferably at least 85%, more preferably at least 90%, and most preferably at least 95%. Identity or similarity with respect to this sequence is defined herein as the percentage of amino acid residues in the candidate sequence that are identical (i.e same residue) or similar (i.e. amino acid residue from the same group based on common side-chain properties, see below) with the species-dependent antibody residues, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. None of N-terminal, C-terminal, or internal extensions, deletions, or insertions into the antibody sequence outside of the variable domain shall be construed as affecting sequence identity or similarity.

To increase the half-life of the antibodies or polypeptide containing the amino acid sequences of this invention, one can attach a salvage receptor binding epitope to the antibody (especially an antibody fragment), as described, e.g., in US Patent 5,739,277. For example, a nucleic acid molecule encoding the salvage receptor binding epitope can be linked in frame to a nucleic acid encoding a polypeptide sequence of this invention so that the fusion protein expressed by the engineered nucleic acid molecule comprises the salvage receptor binding epitope and a polypeptide sequence of this invention. As used herein, the term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (*e.g.,* IgG_1, IgG_2, IgG_3, or IgG_4) that is responsible for increasing the *in vivo* serum half-life of the IgG molecule (e.g., Ghetie et al., *Ann. Rev. Immunol.* 18:739-766 (2000), Table 1). Antibodies with substitutions in an Fc region thereof and increased serum half-lives are also described in

WO00/42072, WO 02/060919; Shields et al., *J. Biol. Chem.* 276:6591-6604 (2001); Hinton, *J. Biol. Chem.* 279:6213-6216 (2004)). In another embodiment, the serum half-life can also be increased, for example, by attaching other polypeptide sequences. For example, antibodies or other polypeptides useful in the methods of the invention can be attached to serum albumin or a portion of serum albumin that binds to the FcRn receptor or a serum albumin binding peptide so that serum albumin binds to the antibody or polypeptide, e.g., such polypeptide sequences are disclosed in WO01/45746. In one embodiment, the serum albumin peptide to be attached comprises an amino acid sequence of DICLPRWGCLW. In another embodiment, the half-life of a Fab is increased by these methods. *See also*, Dennis et al. *J. Biol. Chem.* 277:35035-35043 (2002) for serum albumin binding peptide sequences.

A "chimeric VEGF receptor protein" is a VEGF receptor molecule having amino acid sequences derived from at least two different proteins, at least one of which is a VEGF receptor protein. In certain embodiments, the chimeric VEGF receptor protein is capable of binding to and inhibiting the biological activity of VEGF.

An "isolated" antibody is one that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In certain embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, silver stain. Isolated antibody includes the antibody in situ within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

By "fragment" is meant a portion of a polypeptide or nucleic acid molecule that contains, preferably, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or more of the entire length of the reference nucleic acid molecule or polypeptide. A fragment may contain 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100, 200, 300, 400, 500, 600, or more nucleotides or 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 120, 140, 160, 180, 190, 200 amino acids or more.

An "anti-angiogenesis agent" or "angiogenesis inhibitor" refers to a small molecular weight substance, a polynucleotide, a polypeptide, an isolated protein, a recombinant protein,

an antibody, or conjugates or fusion proteins thereof, that inhibits angiogenesis, vasculogenesis, or undesirable vascular permeability, either directly or indirectly. It should be understood that the anti-angiogenesis agent includes those agents that bind and block the angiogenic activity of the angiogenic factor or its receptor. For example, an anti-angiogenesis agent is an antibody or other antagonist to an angiogenic agent as defined throughout the specification or known in the art, e.g., but are not limited to, antibodies to VEGF-A or to the VEGF-A receptor (e.g., KDR receptor or Flt-1 receptor), VEGF-trap, anti-PDGFR inhibitors such as Gleevec™ (Imatinib Mesylate). Anti-angiogensis agents also include native angiogenesis inhibitors, e.g., angiostatin, endostatin, etc. *See, e.g.*, Klagsbrun and D'Amore, *Annu. Rev. Physiol.*, 53:217-39 (1991); Streit and Detmar, *Oncogene*, 22:3172-3179 (2003) (e.g., Table 3 listing anti-angiogenic therapy in malignant melanoma); Ferrara & Alitalo, *Nature Medicine* 5:1359-1364 (1999); Tonini et al., *Oncogene*, 22:6549-6556 (2003) (e.g., Table 2 listing known antiangiogenic factors); and Sato. *Int. J. Clin. Oncol.*, 8:200-206 (2003) (e.g., Table 1 lists anti-angiogenic agents used in clinical trials).

A "maintenance" dose herein refers to one or more doses of a therapeutic agent administered to the subject over or after a treatment period. Usually, the maintenance doses are administered at spaced treatment intervals, such as approximately every week, approximately every 2 weeks, approximately every 3 weeks, or approximately every 4 weeks.

"Survival" refers to the subject remaining alive, and includes progression free survival (PFS) and overall survival (OS). Survival can be estimated by the Kaplan-Meier method, and any differences in survival are computed using the stratified log-rank test.

"Progression free survival (PFS)" refers to the time from treatment (or randomization) to first disease progression or death. For example it is the time that the subject remains alive, without return of the cancer, e.g., for a defined period of time such as about 1 month, 1.2 months, 2 months, 2.4 months, 2.9 months, 3 months, 3.5 months, 4, months, 6 months, 7 months, 8 months, 9 months, 1 year, about 2 years, about 3 years, etc., from initiation of treatment or from initial diagnosis. In one embodiment, the PFS is extended about 2.9 months to 3.5 months (e.g., with capecitabine). In one embodiment, the PFS is extended about 1.2 months to about 2.4 months (e.g., with taxane/anthracycline). In one aspect of the invention, PFS can be assessed by Response Evaluation Criteria in Solid Tumors (RECIST).

"Overall survival" refers to the subject remaining alive for a defined period of time, such as about 1 year, about 2 years, about 3 years, about 4 years, about 5 years, about 10 years, etc., from initiation of treatment or from initial diagnosis. In the studies underlying the invention the event used for survival analysis was death from any cause.

By "extending survival" or "increasing the likelihood of survival" is meant increasing PFS and/or OS in a treated subject relative to an untreated subject (i.e. relative to a subject not treated with a VEGF-specific antagonist, e.g., a VEGF antibody), or relative to a control treatment protocol, such as treatment only with the chemotherapeutic agent, such as those use in the standard of care for breast cancer, e.g., capecitabine, taxane, anthracycline, paclitaxel, docetaxel, paclitaxel protein-bound particles (e.g., Abraxane®), doxorubicin, epirubicin, 5-fluorouracil, cyclophosphamide or combinations thereof. Survival is monitored for at least about one month, two months, four months, six months, nine months, or at least about 1 year, or at least about 2 years, or at least about 3 years, or at least about 4 years, or at least about 5 years, or at least about 10 years, etc., following the initiation of treatment or following the initial diagnosis.

Hazard ratio (HR) is a statistical definition for rates of events. For the purpose of the invention, hazard ratio is defined as representing the probability of an event in the experimental arm divided by the probability of an event in the control arm at any specific point in time. "Hazard ratio" in progression free survival analysis is a summary of the difference between two progression free survival curves, representing the reduction in the risk of death on treatment compared to control, over a period of follow-up.

The term "concurrently" is used herein to refer to administration of two or more therapeutic agents, where at least part of the administration overlaps in time. Accordingly, concurrent administration includes a dosing regimen when the administration of one or more agent(s) continues after discontinuing the administration of one or more other agent(s).

By "monotherapy" is meant a therapeutic regimen that includes only a single therapeutic agent for the treatment of the cancer or tumor during the course of the treatment period. Monotherapy using a VEGF-specific antagonist means that the VEGF-specific antagonist is administered in the absence of an additional anti-cancer therapy during treatment period.

By "maintenance therapy" is meant a therapeutic regimen that is given to reduce the likelihood of disease recurrence or progression. Maintenance therapy can be provided for any length of time, including extended time periods up to the life-span of the subject. Maintenance therapy can be provided after initial therapy or in conjunction with initial or additional therapies. Dosages used for maintenance therapy can vary and can include diminished dosages as compared to dosages used for other types of therapy. See also "maintenance" herein.

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Included in this definition are benign and malignant cancers as well as dormant tumors or micrometastatses. Examples of cancer include but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include breast cancer, squamous cell cancer, lung cancer (including small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, and squamous carcinoma of the lung), cancer of the peritoneum, hepatocellular cancer, gastric or stomach cancer (including gastrointestinal cancer), pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, colon cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney or renal cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer, as well as B-cell lymphoma (including low grade/follicular non-Hodgkin's lymphoma (NHL); small lymphocytic (SL) NHL; intermediate grade/follicular NHL; intermediate grade diffuse NHL; high grade immunoblastic NHL; high grade lymphoblastic NHL; high grade small non-cleaved cell NHL; bulky disease NHL; mantle cell lymphoma; AIDS-related lymphoma; and Waldenstrom's Macroglobulinemia); chronic lymphocytic leukemia (CLL); acute lymphoblastic leukemia (ALL); Hairy cell leukemia; chronic myeloblastic leukemia; and post-transplant lymphoproliferative disorder (PTLD), as well as abnormal vascular proliferation associated with phakomatoses, edema (such as that associated with brain tumors), and Meigs' syndrome.

By "metastasis" is meant the spread of cancer from its primary site to other places in the body. Cancer cells can break away from a primary tumor, penetrate into lymphatic and blood vessels, circulate through the bloodstream, and grow in a distant focus (metastasize) in normal tissues elsewhere in the body. Metastasis can be local or distant. Metastasis is a sequential process, contingent on tumor cells breaking off from the primary tumor, traveling through the bloodstream, and stopping at a distant site. At the new site, the cells establish a blood supply and can grow to form a life-threatening mass. Both stimulatory and inhibitory molecular pathways within the tumor cell regulate this behavior, and interactions between the tumor cell and host cells in the distant site are also significant.

By "subject" is meant a mammal, including, but not limited to, a human or non-human mammal, such as a bovine, equine, canine, ovine, or feline. Preferably, the subject is a human. Patients are also subjects herein.

For the methods of the present invention, the term "instructing" a subject means providing directions for applicable therapy, medication, treatment, treatment regimens, and

the like, by any means, but preferably in writing, such as in the form of package inserts or other written promotional material.

For the methods of the present invention, the term "promoting" means offering, advertising, selling, or describing a particular drug, combination of drugs, or treatment modality, by any means, including writing, such as in the form of package inserts. Promoting herein refers to promotion of a therapeutic agent, such as a VEGF antagonist, e.g., anti-VEGF antibody or chemotherapeutic agent, for an indication, such as breast cancer treatment, where such promoting is authorized by the Food and Drug Administration (FDA) as having been demonstrated to be associated with statistically significant therapeutic efficacy and acceptable safety in a population of subjects.

The term "marketing" is used herein to describe the promotion, selling or distribution of a product (*e.g.,* drug). Marketing specifically includes packaging, advertising, and any business activity with the purpose of commercializing a product.

A "population" of subjects refers to a group of subjects with cancer, such as in a clinical trial, or as seen by oncologists following FDA approval for a particular indication, such as breast cancer therapy. In one embodiment, the population comprises at least about 1200 subjects.

The term "anti-cancer therapy" refers to a therapy useful in treating cancer. Examples of anti-cancer therapeutic agents include, but are limited to, e.g., surgery, chemotherapeutic agents, growth inhibitory agents, cytotoxic agents, agents used in radiation therapy, anti-angiogenesis agents, apoptotic agents, anti-tubulin agents, and other agents to treat cancer, such as anti-HER-2 antibodies (e.g., Herceptin®), anti-CD20 antibodies, an epidermal growth factor receptor (EGFR) antagonist (e.g., a tyrosine kinase inhibitor), HER1/EGFR inhibitor (e.g., erlotinib (Tarceva®)), platelet derived growth factor inhibitors (e.g., Gleevec™ (Imatinib Mesylate)), a COX-2 inhibitor (e.g., celecoxib), interferons, cytokines, antagonists (e.g., neutralizing antibodies) that bind to one or more of the following targets ErbB2, ErbB3, ErbB4, PDGFR-beta, BlyS, APRIL, BCMA or VEGF receptor(s), TRAIL/Apo2, and other bioactive and organic chemical agents, etc. Combinations thereof are also included in the invention.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (*e.g.* At^{211}, I^{131}, I^{125}, Y^{90}, Re^{186}, Re^{188}, Sm^{153}, Bi^{212}, P^{32} and radioactive isotopes of Lu), chemotherapeutic agents, and toxins such as small molecule toxins or

enzymatically active toxins of bacterial, fungal, plant or animal origin, including fragments and/or variants thereof.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and CYTOXAN® cyclosphosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethiylenethiophosphoramide and trimethylolomelamine; acetogenins (especially bullatacin and bullatacinone); a camptothecin (including the synthetic analogue topotecan); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB1-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimnustine; antibiotics such as the enediyne antibiotics (e. g., calicheamicin, especially calicheamicin gamma1I and calicheamicin omegaI1 (see, e.g., Agnew, *Chem Intl. Ed. Engl.*, 33: 183-186 (1994)); dynemicin, including dynemicin A; bisphosphonates, such as clodronate; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antiobiotic chromophores), aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabicin, carminomycin, carzinophilin, chromomycinis, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, ADRIAMYCIN® doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine; androgens such as calusterone, dromostanolone propionate,

epitiostanol, mepitiostane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elfornithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; podophyllinic acid; 2- ethylhydrazide; procarbazine; PSK® polysaccharide complex (JHS Natural Products, Eugene, OR); razoxane; rhizoxin; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, e.g., TAXOL® paclitaxel (Bristol- Myers Squibb Oncology, Princeton, N.J.), ABRAXANE® Cremophor-free, albumin-engineered nanoparticle formulation of paclitaxel (American Pharmaceutical Partners, Schaumberg, Illinois), and TAXOTERE® doxetaxel (Rhône- Poulenc Rorer, Antony, France); chloranbucil; GEMZAR® gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin, oxaliplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitoxantrone; vincristine; NAVELBINE® vinorelbine; novantrone; teniposide; edatrexate; daunomycin; aminopterin; xeloda; ibandronate; irinotecan (Camptosar, CPT-11) (including the treatment regimen of irinotecan with 5-FU and leucovorin); topoisomerase inhibitor RFS 2000; difluorometlhylornithine (DMFO); retinoids such as retinoic acid; capecitabine; combretastatin; leucovorin (LV); oxaliplatin, including the oxaliplatin treatment regimen (FOLFOX); lapatinib (Tykerb®); inhibitors of PKC-alpha, Raf, H-Ras, EGFR (e.g., erlotinib (Tarceva®)) and VEGF-A that reduce cell proliferation and pharmaceutically acceptable salts, acids or derivatives of any of the above.

Also included in this definition are anti-hormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens and selective estrogen receptor modulators (SERMs), including, for example, tamoxifen (including NOLVADEX® tamoxifen), raloxifene, droloxifene, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and FARESTON· toremifene; aromatase inhibitors that inhibit the enzyme aromatase, which regulates estrogen production in the adrenal glands, such as, for example, 4(5)-imidazoles, aminoglutethimide, MEGASE® megestrol acetate, AROMASIN® exemestane, formestanie, fadrozole, RIVISOR® vorozole, FEMARA® letrozole, and ARIMIDEX® anastrozole; and anti-androgens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; as well

as troxacitabine (a 1,3-dioxolane nucleoside cytosine analog); antisense oligonucleotides, particularly those which inhibit expression of genes in signaling pathways implicated in abherant cell proliferation, such as, for example, PKC-alpha, Ralf and H-Ras; ribozymes such as a VEGF expression inhibitor (e.g., ANGIOZYME® ribozyme) and a HER2 expression inhibitor; vaccines such as gene therapy vaccines, for example, ALLOVECTIN® vaccine, LEUVECTIN® vaccine, and VAXID® vaccine; PROLEUKIN® rIL-2; LURTOTECAN® topoisomerase 1 inhibitor; ABARELIX® rmRH; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

The term "cytokine" is a generic term for proteins released by one cell population which act on another cell as intercellular mediators. Examples of such cytokines are lymphokines, monokines, and traditional polypeptide hormones. Included among the cytokines are growth hormone such as human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); epidermal growth factor; hepatic growth factor; fibroblast growth factor; prolactin; placental lactogen; tumor necrosis factor-alpha and -beta; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors such as NGF-alpha; platelet-growth factor; transforming growth factors (TGFs) such as TGF-alpha and TGF-beta; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon-alpha, -beta and -gamma colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as IL-1, IL-1alpha, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12; a tumor necrosis factor such as TNF-alpha or TNF-beta; and other polypeptide factors including LIF and kit ligand (KL). As used herein, the term cytokine includes proteins from natural sources or from recombinant cell culture and biologically active equivalents of the native sequence cytokines.

A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell in vitro and/or in vivo. Thus, the growth inhibitory agent may be one which significantly reduces the percentage of cells in S phase. Examples of growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and vinblastine), TAXOL®, and topo II inhibitors such as doxorubicin,

epirubicin, daunorubicin, etoposide, and bleomycin. Those agents that arrest G1 also spill over into S-phase arrest, for example, DNA alkylating agents such as tamoxifen, prednisone, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, and ara-C. Further information can be found in The Molecular Basis of Cancer, Mendelsohn and Israel, eds., Chapter 1, entitled "Cell cycle regulation, oncogenes, and antineoplastic drugs" by Murakami et al. (WB Saunders: Philadelphia, 1995), especially p. 13.

The term "prodrug" as used in this application refers to a precursor or derivative form of a pharmaceutically active substance that is less cytotoxic to tumor cells compared to the parent drug and is capable of being enzymatically activated or converted into the more active parent form. See, e.g., Wilman, "Prodrugs in Cancer Chemotherapy" *Biochemical Society Transactions*, 14, pp. 375-382, 615th Meeting Belfast (1986) and Stella et al., "Prodrugs: A Chemical Approach to Targeted Drug Delivery," *Directed Drug Delivery*, Borchardt et al., (ed.), pp. 247-267, Humana Press (1985). The prodrugs of this invention include, but are not limited to, phosphate-containing prodrugs, thiophosphate-containing prodrugs, sulfate-containing prodrugs, peptide-containing prodrugs, D-amino acid-modified prodrugs, glycosylated prodrugs, β-lactam-containing prodrugs, optionally substituted phenoxyacetamide-containing prodrugs or optionally substituted phenylacetamide-containing prodrugs, 5-fluorocytosine and other 5-fluorouridine prodrugs which can be converted into the more active cytotoxic free drug. Examples of cytotoxic drugs that can be derivatized into a prodrug form for use in this invention include, but are not limited to, those chemotherapeutic agents described above.

By "radiation therapy" is meant the use of directed gamma rays or beta rays to induce sufficient damage to a cell so as to limit its ability to function normally or to destroy the cell altogether. It will be appreciated that there will be many ways known in the art to determine the dosage and duration of treatment. Typical treatments are given as a one time administration and typical dosages range from 10 to 200 units (Grays) per day.

By "reduce or inhibit" is meant the ability to cause an overall decrease preferably of 20% or greater, more preferably of 50% or greater, and most preferably of 75%, 85%, 90%, 95%, or greater. Reduce or inhibit can refer to the symptoms of the disorder being treated, the presence or size of metastases or micrometastases, the size of the primary tumor, the presence or the size of the dormant tumor, or the size or number of the blood vessels in angiogenic disorders.

The term "intravenous infusion" refers to introduction of a drug into the vein of an animal or human subject over a period of time greater than approximately 5 minutes, preferably between

approximately 30 to 90 minutes, although, according to the invention, intravenous infusion is alternatively administered for 10 hours or less.

The term "intravenous bolus" or "intravenous push" refers to drug administration into a vein of an animal or human such that the body receives the drug in approximately 15 minutes or less, preferably 5 minutes or less.

The term "subcutaneous administration" refers to introduction of a drug under the skin of an animal or human subject, preferable within a pocket between the skin and underlying tissue, by relatively slow, sustained delivery from a drug receptacle. The pocket may be created by pinching or drawing the skin up and away from underlying tissue.

The term "subcutaneous infusion" refers to introduction of a drug under the skin of an animal or human subject, preferably within a pocket between the skin and underlying tissue, by relatively slow, sustained delivery from a drug receptacle for a period of time including, but not limited to, 30 minutes or less, or 90 minutes or less. Optionally, the infusion may be made by subcutaneous implantation of a drug delivery pump implanted under the skin of the animal or human subject, wherein the pump delivers a predetermined amount of drug for a predetermined period of time, such as 30 minutes, 90 minutes, or a time period spanning the length of the treatment regimen.

The term "subcutaneous bolus" refers to drug administration beneath the skin of an animal or human subject, where bolus drug delivery is preferably less than approximately 15 minutes, more preferably less than 5 minutes, and most preferably less than 60 seconds. Administration is preferably within a pocket between the skin and underlying tissue, where the pocket is created, for example, by pinching or drawing the skin up and away from underlying tissue.

A "disorder" is any condition that would benefit from treatment with the antibody. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. Non-limiting examples of disorders to be treated herein include cancer; benign and malignant tumors; leukemias and lymphoid malignancies; neuronal, glial, astrocytal, hypothalamic and other glandular, macrophagal, epithelial, stromal and blastocoelic disorders; and inflammatory, angiogenic and immunologic disorders.

The term "therapeutically effective amount" refers to an amount of a drug effective to treat a disease or disorder in a mammal. In the case of cancer, the therapeutically effective amount of the drug may reduce the number of cancer cells; reduce the tumor size; inhibit (*i.e.*,

slow to some extent and preferably stop) cancer cell infiltration into peripheral organs; inhibit (*i.e.*, slow to some extent and preferably stop) tumor metastasis; inhibit, to some extent, tumor growth; and/or relieve to some extent one or more of the symptoms associated with the disorder. To the extent the drug may prevent growth and/or kill existing cancer cells, it may be cytostatic and/or cytotoxic. For cancer therapy, efficacy *in vivo* can, for example, be measured by assessing the duration of survival, duration of progression free survival (PFS), the response rates (RR), duration of response, and/or quality of life.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

The word "label" when used herein refers to a detectable compound or composition which is conjugated directly or indirectly to the polypeptide. The label may be itself be detectable (*e.g.,* radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition which is detectable.

II. ANTI-VEGF ANTIBODIES AND ANTAGONISTS

(i) VEGF Antigen

The VEGF antigen to be used for production of antibodies may be, e.g., the $VEGF_{165}$ molecule as well as other isoforms of VEGF or a fragment thereof containing the desired epitope. Other forms of VEGF useful for generating anti-VEGF antibodies of the invention will be apparent to those skilled in the art.

Human VEGF was obtained by first screening a cDNA library prepared from human cells, using bovine VEGF cDNA as a hybridization probe. Leung *et al.* (1989) *Science*, 246:1306. One cDNA identified thereby encodes a 165-amino acid protein having greater than 95% homology to bovine VEGF; this 165-amino acid protein is typically referred to as human VEGF (hVEGF) or $VEGF_{165}$. The mitogenic activity of human VEGF was confirmed by expressing the human VEGF cDNA in mammalian host cells. Media conditioned by cells transfected with the human VEGF cDNA promoted the proliferation of capillary endothelial cells, whereas control cells did not. Leung *et al.* (1989) *Science, supra.* Further efforts were undertaken to clone and express VEGF via recombinant DNA techniques. (*See, e.g.*, Ferrara, *Laboratory Investigation* 72:615-618 (1995), and the references cited therein).

VEGF is expressed in a variety of tissues as multiple homodimeric forms (121, 145, 165, 189, and 206 amino acids per monomer) resulting from alternative RNA splicing. $VEGF_{121}$ is a soluble mitogen that does not bind heparin; the longer forms of VEGF bind heparin with progressively higher affinity. The heparin-binding forms of VEGF can be cleaved in the carboxy terminus by plasmin to release a diffusible form(s) of VEGF. Amino acid sequencing of the carboxy terminal peptide identified after plasmin cleavage is Arg_{110}-Ala_{111}. Amino terminal "core" protein, VEGF (1-110) isolated as a homodimer, binds neutralizing monoclonal antibodies (such as the antibodies referred to as 4.6.1 and 3.2E3.1.1) and soluble forms of VEGF receptors with similar affinity compared to the intact $VEGF_{165}$ homodimer.

Several molecules structurally related to VEGF have also been identified recently, including placenta growth factor (PIGF), VEGF-B, VEGF-C, VEGF-D and VEGF-E. Ferrara and Davis-Smyth (1987) Endocr. Rev., *supra*; Ogawa *et al. J. Biological Chem.* 273:31273-31281(1998); Meyer *et al. EMBO J.*, 18:363-374(1999). A receptor tyrosine kinase, Flt-4 (VEGFR-3), has been identified as the receptor for VEGF-C and VEGF-D. Joukov et al. *EMBO. J.* 15:1751(1996); Lee et al. *Proc. Natl. Acad. Sci.* USA 93:1988-1992(1996); Achen et al. (1998) *Proc. Natl. Acad. Sci.* USA 95:548-553. VEGF-C has been shown to be involved in the regulation of lymphatic angiogenesis. Jeltsch et al. *Science* 276:1423-1425(1997).

Two VEGF receptors have been identified, Flt-1 (also called VEGFR-1) and KDR (also called VEGFR-2). Shibuya et al. (1990) *Oncogene* 8:519-527; de Vries et al. (1992) *Science* 255:989-991; Terman et al. (1992) *Biochem. Biophys. Res. Commun.* 187:1579-1586. Neuropilin-1 has been shown to be a selective VEGF receptor, able to bind the heparin-binding VEGF isoforms (Soker et al. (1998) *Cell* 92:735-45).

(ii) Anti-VEGF Antibodies

Anti-VEGF antibodies that are useful in the methods of the invention include any antibody, or antigen binding fragment thereof, that bind with sufficient affinity and specificity to VEGF and can reduce or inhibit the biological activity of VEGF. An anti-VEGF antibody will usually not bind to other VEGF homologues such as VEGF-B or VEGF-C, nor other growth factors such as PlGF, PDGF, or bFGF.

In certain embodiments of the invention, the anti-VEGF antibodies include, but are not limited to, a monoclonal antibody that binds to the same epitope as the monoclonal anti-VEGF antibody A4.6.1 produced by hybridoma ATCC HB 10709; a recombinant humanized anti-VEGF monoclonal antibody generated according to Presta et al. (1997) Cancer Res. 57:4593-4599. In one embodiment, the anti-VEGF antibody is "Bevacizumab (BV)", also known as

"rhuMAb VEGF" or "AVASTIN®". It comprises mutated human IgG1 framework regions and antigen-binding complementarity-determining regions from the murine anti-hVEGF monoclonal antibody A.4.6.1 that blocks binding of human VEGF to its receptors. Approximately 93% of the amino acid sequence of bevacizumab, including most of the framework regions, is derived from human IgG1, and about 7% of the sequence is derived from the murine antibody A4.6.1.

Bevacizumab (AVASTIN®) was the first anti-angiogenesis therapy approved by the FDA and is approved for the treatment metastatic colorectal cancer (first- and second-line treatment in combination with intravenous 5-FU-based chemotherapy), advanced non-squamous, non-small cell lung cancer (NSCLC) (first-line treatment of unresectable, locally advanced, recurrent or metastatic NSCLC in combination with carboplatin and paclitaxel) and metastatic HER2-negative breast cancer (previously untreated, metastatic HER2-negative breast cancer in combination with paclitaxel).

Bevacizumab and other humanized anti-VEGF antibodies are further described in U.S. Pat. No. 6,884,879 issued Feb. 26, 2005. Additional antibodies include the G6 or B20 series antibodies (e.g., G6-31, B20-4.1), as described in PCT Publication No. WO2005/012359, PCT Publication No. WO2005/044853, and US Patent Application 60/991,302, the content of these patent applications are expressly incorporated herein by reference. For additional antibodies see U.S. Pat. Nos. 7,060,269, 6,582,959, 6,703,020; 6,054,297; WO98/45332; WO 96/30046; WO94/10202; EP 0666868B1; U.S. Patent Application Publication Nos. 2006009360, 20050186208, 20030206899, 20030190317, 20030203409, and 20050112126; and Popkov et al., Journal of Immunological Methods 288:149-164 (2004). Other antibodies include those that bind to a functional epitope on human VEGF comprising of residues F17, M18, D19, Y21, Y25, Q89, I91, K101, E103, and C104 or, alternatively, comprising residues F17, Y21, Q22, Y25, D63, I83 and Q89.

In one embodiment of the invention, the anti-VEGF antibody has a heavy chain variable region comprising the following amino acid sequence:

EVQLVESGGG LVQPGGSLRL SCAASGYTFT NYGMNWVRQA PGKGLEWVGW

INTYTGEPTY AADFKRRFTF SLDTSKSTAY LQMNSLRAED TAVYYCAKYP

HYYGSSHWYF DVWGQGTLVT VSS (SEQ ID No. 1)

and a light chain variable region comprising the following amino acid sequence:

DIQMTQSPSS LSASVGDRVT ITCSASQDIS NYLNWYQQKP GKAPKVLIYF

TSSLHSGVPS RFSGSGSGTD FTLTISSLQP EDFATYYCQQ YSTVPWTFGQ

GTKVEIKR (SEQ ID No. 2).

A "G6 series antibody" according to this invention, is an anti-VEGF antibody that is derived from a sequence of a G6 antibody or G6-derived antibody according to any one of Figures 7, 24-26, and 34-35 of PCT Publication No. WO2005/012359, the entire disclosure of which is expressly incorporated herein by reference. See also PCT Publication No. WO2005/044853, the entire disclosure of which is expressly incorporated herein by reference. In one embodiment, the G6 series antibody binds to a functional epitope on human VEGF comprising residues F17, Y21, Q22, Y25, D63, I83 and Q89.

A "B20 series antibody" according to this invention is an anti-VEGF antibody that is derived from a sequence of the B20 antibody or a B20-derived antibody according to any one of Figures 27-29 of PCT Publication No. WO2005/012359, the entire disclosure of which is expressly incorporated herein by reference. See also PCT Publication No. WO2005/044853, and US Patent Application 60/991,302, the content of these patent applications are expressly incorporated herein by reference. In one embodiment, the B20 series antibody binds to a functional epitope on human VEGF comprising residues F17, M18, D19, Y21, Y25, Q89, I91, K101, E103, and C104.

A "functional epitope" according to this invention refers to amino acid residues of an antigen that contribute energetically to the binding of an antibody. Mutation of any one of the energetically contributing residues of the antigen (for example, mutation of wild-type VEGF by alanine or homolog mutation) will disrupt the binding of the antibody such that the relative affinity ratio (IC50mutant VEGF/IC50wild-type VEGF) of the antibody will be greater than 5 (see Example 2 of WO2005/012359). In one embodiment, the relative affinity ratio is determined by a solution binding phage displaying ELISA. Briefly, 96-well Maxisorp immunoplates (NUNC) are coated overnight at 4°C with an Fab form of the antibody to be tested at a concentration of 2ug/ml in PBS, and blocked with PBS, 0.5% BSA, and 0.05% Tween20 (PBT) for 2h at room temperature. Serial dilutions of phage displaying hVEGF alanine point mutants (residues 8-109 form) or wild type hVEGF (8-109) in PBT are first incubated on the Fab-coated plates for 15 min at room temperature, and the plates are washed with PBS, 0.05% Tween20 (PBST). The bound phage is detected with an anti-M13 monoclonal antibody horseradish peroxidase (Amersham Pharmacia) conjugate diluted 1:5000

in PBT, developed with 3,3', 5,5'-tetramethylbenzidine (TMB, Kirkegaard & Perry Labs, Gaithersburg, MD) substrate for approximately 5 min, quenched with 1.0 M H3PO4, and read spectrophotometrically at 450 nm. The ratio of lC50 values (lC50,ala/lC50,wt) represents the fold of reduction in binding affinity (the relative binding affinity).

(iii) VEGF receptor molecules

The two best characterized VEGF receptors are VEGFR1 (also known as Flt-1) and VEGFR2 (also known as KDR and FLK-1 for the murine homolog). The specificity of each receptor for each VEGF family member varies but VEGF-A binds to both Flt-1 and KDR. Both Flt-I and KDR belong to the family of receptor tyrosine kinases (RTKs). The RTKs comprise a large family of transmembrane receptors with diverse biological activities. At least nineteen (19) distinct RTK subfamilies have been identified. The receptor tyrosine kinase (RTK) family includes receptors that are crucial for the growth and differentiation of a variety of cell types (Yarden and Ullrich (1988) *Ann. Rev. Biochem.* 57:433-478; Ullrich and Schlessinger (1990) *Cell* 61:243-254). The intrinsic function of RTKs is activated upon ligand binding, which results in phosphorylation of the receptor and multiple cellular substrates, and subsequently in a variety of cellular responses (Ullrich & Schlessinger (1990) *Cell* 61:203-212). Thus, receptor tyrosine kinase mediated signal transduction is initiated by extracellular interaction with a specific growth factor (ligand), typically followed by receptor dimerization, stimulation of the intrinsic protein tyrosine kinase activity and receptor trans-phosphorylation. Binding sites are thereby created for intracellular signal transduction molecules and lead to the formation of complexes with a spectrum of cytoplasmic signaling molecules that facilitate the appropriate cellular response. (e.g., cell division, differentiation, metabolic effects, changes in the extracellular microenvironment) see, Schlessinger and Ullrich (1992) *Neuron* 9:1-20. Structurally, both Flt-1 and KDR have seven immunoglobulin-like domains in the extracellular domain, a single transmembrane region, and a consensus tyrosine kinase sequence which is interrupted by a kinase-insert domain. Matthews et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:9026-9030; Terman et al. (1991) *Oncogene* 6:1677-1683. The extracellular domain is involved in the binding of VEGF and the intracellular domain is involved in signal transduction.

VEGF receptor molecules, or fragments thereof, that specifically bind to VEGF can be used in the methods of the invention to bind to and sequester the VEGF protein, thereby preventing it from signaling. In certain embodiments, the VEGF receptor molecule, or VEGF binding fragment thereof, is a soluble form, such as sFlt-1. A soluble form of the receptor exerts an inhibitory effect on the biological activity of the VEGF protein by binding to VEGF,

thereby preventing it from binding to its natural receptors present on the surface of target cells. Also included are VEGF receptor fusion proteins, examples of which are described below.

A chimeric VEGF receptor protein is a receptor molecule having amino acid sequences derived from at least two different proteins, at least one of which is a VEGF receptor protein (e.g., the flt-1 or KDR receptor), that is capable of binding to and inhibiting the biological activity of VEGF. In certain embodiments, the chimeric VEGF receptor proteins of the invention consist of amino acid sequences derived from only two different VEGF receptor molecules; however, amino acid sequences comprising one, two, three, four, five, six, or all seven Ig-like domains from the extracellular ligand-binding region of the flt-1 and/or KDR receptor can be linked to amino acid sequences from other unrelated proteins, for example, immunoglobulin sequences. Other amino acid sequences to which Ig-like domains are combined will be readily apparent to those of ordinary skill in the art. Examples of chimeric VEGF receptor proteins include, e.g., soluble Flt-1/Fc, KDR/Fc, or FLt-1/KDR/Fc (also known as VEGF Trap). (See for example PCT Application Publication No. WO97/44453)

A soluble VEGF receptor protein or chimeric VEGF receptor proteins of the invention includes VEGF receptor proteins which are not fixed to the surface of cells via a transmembrane domain. As such, soluble forms of the VEGF receptor, including chimeric receptor proteins, while capable of binding to and inactivating VEGF, do not comprise a transmembrane domain and thus generally do not become associated with the cell membrane of cells in which the molecule is expressed.

III. THERAPEUTIC USES AND COMPOSITIONS OF ANTI-VEGF ANTIBODIES

The invention encompasses antiangiogenic therapy, a novel cancer treatment strategy aimed at inhibiting the development of tumor blood vessels required for providing nutrients to support tumor growth. Because angiogenesis is involved in both primary tumor growth and metastasis, the antiangiogenic treatment provided by the invention is capable of inhibiting the neoplastic growth of tumor at the primary site as well as preventing metastasis of tumors at the secondary sites, therefore allowing attack of the tumors by other therapeutics.

Specifically, provided herein are methods of treating a subject diagnosed with previously untreated metastatic breast cancer, comprising administering to the subject a treatment regimen combining an effective amount of at least one chemotherapeutic agent and an anti-VEGF antibody, wherein said subject has not received any chemotherapy for locally recurrent or metastatic breast cancer. Optionally, the subject has not received prior adjuvant

chemotherapy in recurrence less than or equal to 12 months since last dose. The treatment regimen combining the chemotherapy and the administration of the anti-VEGF effectively extends the progression free survival (PFS) of the subject. Further provided herein are uses of an anti-VEGF antibody with at least one chemotherapeutic agent in the manufacturer of a medicament for treating previously untreated metastatic breast cancer in a subject, wherein said subject has not received any chemotherapy for locally recurrent or metastatic breast cancer. Optionally, the subject has not received prior adjuvant chemotherapy in recurrence less than or equal to 12 months since last dose. The use of the anti-VEGF and the chemotherapeutic agent effectively extends the progression free survival (PFS) of the subject. Provided also herein are anti-VEGF antibodies for use in a method of treating locally recurrent or metastatic breast cancer in a subject, the method comprising administering to the subject a treatment regimen combining an effective amount of at least one chemotherapeutic agent and an anti-VEGF antibody, wherein said subject has not received any chemotherapy for locally recurrent or metastatic breast cancer. Optionally, the subject has not received prior adjuvant chemotherapy in recurrence less than or equal to 12 months since last dose. The use of the anti-VEGF and the chemotherapeutic agent effectively extends the progression free survival (PFS) of the subject. In certain embodiments, in any of the methods, uses, and compositions of the invention, the administration of the chemotherapy and the anti-VEGF antibody has a safety profile that is consistent with results of prior bevacizumab trials (see, e.g., the bevacizumab product insert).

In some embodiments of the invention, the subject is HER2 negative. In some embodiments of the invention, the subject is HER2 positive. HER2 is recognized as an important predictive and prognostic factor in some breast cancers. *See, e.g.,* Slamon DJ, et al. *Science*. 1989;244:707-712; and Sjögren S, et al. *J Clin Oncol*. 1998;16:462-469. HER2 gene amplification is a permanent genetic change that results in the continuous overexpression of the HER2 receptor (HER2 protein). *See, e.g.,* Simon R, et al. *J Natl Cancer Inst*. 2001;93:1141-11465; and, Sliwkowski MX, et al. *Semin Oncol*. 1999;26(suppl 12):60-70. Several studies have shown that HER2 overexpression (either extra copies of the gene itself, or an excess amount of the gene's protein product) is associated with decreased overall survival. *See, e.g.,* Slamon DJ, et al. *Science*. 1987;235:177-182; and, Paik S, et al. *J Clin Oncol*. 1990;8:103-112. Several commercial assays are available to determine HER2 status, e.g., HercepTest® and Pathway™ for protein and PathVysion® and HER2 FISH pharmDx™ for gene alteration.

Combination Therapies

The invention features the use or compositions of a combination of at least one VEGF-specific antagonist with one or more additional anti-cancer therapies. Examples of anti-cancer therapies include, without limitation, surgery, radiation therapy (radiotherapy), biotherapy, immunotherapy, chemotherapy, or a combination of these therapies. In addition, cytotoxic agents, anti-angiogenic and anti-proliferative agents can be used in combination with the VEGF-specific antagonist.

In certain aspects of any of the methods and uses, the invention provides treating breast cancer, by administering effective amounts of an anti-VEGF antibody and one or more chemotherapeutic agents to a subject susceptible to, or diagnosed with, locally recurrent or previously untreated metastatic cancer. A variety of chemotherapeutic agents may be used in the combined treatment methods and uses of the invention. An exemplary and non-limiting list of chemotherapeutic agents contemplated is provided herein under "Definition", or described herein.

In one example, the invention features the methods and uses of a VEGF-specific antagonist with one or more chemotherapeutic agents (e.g., a cocktail) or any combination thereof. In certain embodiments, the chemotherapeutic agent is for example, capecitabine, taxane, anthracycline, paclitaxel, docetaxel, paclitaxel protein-bound particles (e.g., Abraxane®), doxorubicin, epirubicin, 5-fluorouracil, cyclophosphamide or combinations thereof therapy. In certain embodiments, VEGF antagonist (e.g., anti-VEGF antibody) is combined with lapatinib (Tykerb®). The combined administration includes simultaneous administration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities. Preparation and dosing schedules for such chemotherapeutic agents may be used according to manufacturers' instructions or as determined empirically by the skilled practitioner. Preparation and dosing schedules for chemotherapy are also described in *Chemotherapy Service Ed.*, M. C. Perry, Williams & Wilkins, Baltimore, Md. (1992). The chemotherapeutic agent may precede, or follow administration of the VEGF-specific antagonist or may be given simultaneously therewith.

In some other aspects of any of the methods and uses, other therapeutic agents useful for combination tumor therapy with the antibody of the invention include antagonist of other factors that are involved in tumor growth, such as EGFR, ErbB2 (also known as Her2), ErbB3, ErbB4, or TNF. Sometimes, it may be beneficial to also administer one or more

cytokines to the subject. In one embodiment, the VEGF antibody is co-administered with a growth inhibitory agent. For example, the growth inhibitory agent may be administered first, followed by the VEGF antibody. However, simultaneous administration or administration of the VEGF antibody first is also contemplated. Suitable dosages for the growth inhibitory agent are those presently used and may be lowered due to the combined action (synergy) of the growth inhibitory agent and anti-VEGF antibody.

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. For example, it may be desirable to further provide antibodies which bind to EGFR, VEGF (*e.g.* an antibody which binds a different epitope or same epitope on VEGF), VEGFR, or ErbB2 (e.g., Herceptin®) in the one formulation. Alternatively, or in addition, the composition may comprise a cytotoxic agent, cytokine, growth inhibitory agent and/or VEGFR antagonist. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

In certain aspects of any of the methods and uses, other therapeutic agents useful for combination cancer therapy with the antibody of the invention include other anti-angiogenic agents. Many anti-angiogenic agents have been identified and are known in the arts, including those listed by Carmeliet and Jain (2000). In one embodiment, the anti-VEGF antibody of the invention is used in combination with another VEGF antagonist or a VEGF receptor antagonist such as VEGF variants, soluble VEGF receptor fragments, aptamers capable of blocking VEGF or VEGFR, neutralizing anti-VEGFR antibodies, low molecule weight inhibitors of VEGFR tyrosine kinases and any combinations thereof. Alternatively, or in addition, two or more anti-VEGF antibodies may be co-administered to the subject.

For the prevention or treatment of disease, the appropriate dosage of VEGF-specific antagonist will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the VEGF-specific antagonist is administered for preventive or therapeutic purposes, previous therapy, the subject's clinical history and response to the VEGF-specific antagonist, and the discretion of the attending physician. The VEGF-specific antagonist is suitably administered to the subject at one time or over a series of treatments. In a combination therapy regimen, the VEGF-specific antagonist and the one or more anti-cancer therapeutic agent of the invention are administered in a therapeutically effective or synergistic amount. As used herein, a therapeutically effective amount is such that co-administration of a VEGF-specific antagonist and one or more other therapeutic agents, or administration of a composition of the invention, results in reduction or inhibition of the cancer as described

above. A therapeutically synergistic amount is that amount of a VEGF-specific antagonist and one or more other therapeutic agents necessary to synergistically or significantly reduce or eliminate conditions or symptoms associated with a particular disease.

The VEGF-specific antagonist and the one or more other therapeutic agents can be administered simultaneously or sequentially in an amount and for a time sufficient to reduce or eliminate the occurrence or recurrence of a tumor, a dormant tumor, or a micrometastases. The VEGF-specific antagonist and the one or more other therapeutic agents can be administered as maintenance therapy to prevent or reduce the likelihood of recurrence of the tumor.

As will be understood by those of ordinary skill in the art, the appropriate doses of chemotherapeutic agents or other anti-cancer agents will be generally around those already employed in clinical therapies, e.g., where the chemotherapeutics are administered alone or in combination with other chemotherapeutics. Variation in dosage will likely occur depending on the condition being treated. The physician administering treatment will be able to determine the appropriate dose for the individual subject.

In addition to the above therapeutic regimes, the subject may be subjected to radiation therapy.

In certain embodiments of any of the methods, uses and compositions, the administered VEGF antibody is an intact, naked antibody. However, the VEGF antibody may be conjugated with a cytotoxic agent. In certain embodiments of any of the methods and uses, the conjugated antibody and/or antigen to which it is bound is/are internalized by the cell, resulting in increased therapeutic efficacy of the conjugate in killing the cancer cell to which it binds. In one embodiment, the cytotoxic agent targets or interferes with nucleic acid in the cancer cell. Examples of such cytotoxic agents include maytansinoids, calicheamicins, ribonucleases and DNA endonucleases.

The invention also features a method of instructing a human subject with breast cancer or a health care provider by providing instructions to receive treatment with an anti-VEGF antibody so as to increase the time for progression free survival, to decrease the subject's risk of cancer recurrence or to increase the subject's likelihood of survival. In some embodiments the method further comprises providing instructions to receive treatment with at least one chemotherapeutic agent. The treatment with the anti-VEGF antibody may be concurrent with or sequential to the treatment with the chemotherapeutic agent. In certain embodiments the subject is treated as instructed by the method of instructing. Treatment of breast cancer by

administration of an anti-VEGF antibody with or without chemotherapy may be continued until cancer recurrence or death.

The invention further provides a promotional method, comprising promoting the administration of an anti-VEGF antibody for treatment of breast cancer in a human subject. In some embodiments the method further comprises promoting the administration of at least one chemotherapeutic agent. Administration of the anti-VEGF antibody may be concurrent with or sequential to administration of the chemotherapeutic agent. Promotion may be conducted by any means available. In some embodiments the promotion is by a package insert accompanying a commercial formulation of the anti-VEGF antibody. The promotion may also be by a package insert accompanying a commercial formulation of the chemotherapeutic agent. Promotion may be by written or oral communication to a physician or health care provider. In some embodiments the promotion is by a package insert where the package inset provides instructions to receive breast cancer therapy with anti-VEGF antibody. In a further embodiment, the package insert include some or all of the results under Example 1. In some embodiments the promotion is followed by the treatment of the subject with the anti-VEGF antibody with or without the chemotherapeutic agent.

The invention provides a business method, comprising marketing an anti-VEGF antibody for treatment of breast cancer in a human subject so as to increase the subject's time for progression free survival, to decrease the subject's likelihood of cancer recurrence or increase the subject's likelihood of survival. In some embodiments the method further comprises marketing a chemotherapeutic agent for use in combination with the anti-VEGF antibody. In some embodiments the marketing is followed by treatment of the subject with the anti-VEGF antibody with or without the chemotherapeutic agent.

Also provided is a business method, comprising marketing a chemotherapeutic agent in combination with an anti-VEGF antibody for treatment of breast cancer in a human subject so as to increase the subject's time for progression free survival, to decrease the subject's likelihood of cancer recurrence or increase the subject's likelihood of survival. In some embodiments, the marketing is followed by treatment of the subject with the combination of the chemotherapeutic agent and the anti-VEGF antibody.

IV. DOSAGES AND DURATION

The VEGF-specific antagonist composition will be formulated, dosed, and administered in a fashion consistent with good medical practice. Factors for consideration in this context include the particular disorder being treated, the particular subject being treated,

the clinical condition of the individual subject, the cause of the disorder, the site of delivery of the agent, the method of administration, the scheduling of administration, and other factors known to medical practitioners. The "therapeutically effective amount" of the VEGF-specific antagonist to be administered will be governed by such considerations, and is the minimum amount necessary to prevent, ameliorate, or treat, or stabilize, the cancer; to increase the time until progression (duration of progression free survival) or to treat or prevent the occurrence or recurrence of a tumor, a dormant tumor, or a micrometastases. The VEGF-specific antagonist need not be, but is optionally, formulated with one or more agents currently used to prevent or treat cancer or a risk of developing a cancer. The effective amount of such other agents depends on the amount of VEGF-specific antagonist present in the formulation, the type of disorder or treatment, and other factors discussed above. These are generally used in the same dosages and with administration routes as used hereinbefore or about from 1 to 99% of the heretofore employed dosages.

Depending on the type and severity of the disease, about 1 μg/kg to 100 mg/kg (e.g., 0.1-20 mg/kg) of VEGF-specific antagonist is an initial candidate dosage for administration to the subject, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily dosage might range from about 1 μg/kg to about 100 mg/kg or more, depending on the factors mentioned above. Particularly desirable dosages include, for example, 5 mg/kg, 7.5 mg/kg, 10 mg/kg, and 15 mg/kg. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until the cancer is treated, as measured by the methods described above or known in the art. However, other dosage regimens may be useful. In one example, if the VEGF-specific antagonist is an antibody, the antibody of the invention is administered once every week, every two weeks, or every three weeks, at a dose range from about 5 mg/kg to about 15 mg/kg, including but not limited to 5 mg/kg, 7.5 mg/kg, 10 mg/kg or 15 mg/kg. The progress of the therapy of the invention is easily monitored by conventional techniques and assays. In other embodiments, such dosing regimen is used in combination with a chemotherapy regimen as the first line therapy for treating locally recurrent or metastatic breast cancer. Further information about suitable dosages is provided in the Example below.

The duration of therapy will continue for as long as medically indicated or until a desired therapeutic effect (e.g., those described herein) is achieved. In certain embodiments, the VEGF-specific antagonist therapy is continued for 1 month, 2 months, 4 months, 6 months, 8 months, 10 months, 1 year, 2 years, 3 years, 4 years, 5 years, or for a period of years up to the lifetime of the subject.

The VEGF-specific antagonists of the invention are administered to a subject, e.g., a human subject, in accord with known methods, such as intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. Local administration is particularly desired if extensive side effects or toxicity is associated with the VEGF antagonist. An *ex vivo* strategy can also be used for therapeutic applications. Ex vivo strategies involve transfecting or transducing cells obtained from the subject with a polynucleotide encoding a VEGF antagonist. The transfected or transduced cells are then returned to the subject. The cells can be any of a wide range of types including, without limitation, hematopoietic cells (e.g., bone marrow cells, macrophages, monocytes, dendritic cells, T cells, or B cells), fibroblasts, epithelial cells, endothelial cells, keratinocytes, or muscle cells.

For example, if the VEGF-specific antagonist is an antibody, the antibody is administered by any suitable means, including parenteral, subcutaneous, intraperitoneal, intrapulmonary, and intranasal, and, if desired for local immunosuppressive treatment, intralesional administration. Parenteral infusions include intramuscular, intravenous, intraarterial, intraperitoneal, or subcutaneous administration. In addition, the antibody is suitably administered by pulse infusion, particularly with declining doses of the antibody. Preferably the dosing is given by injections, most preferably intravenous or subcutaneous injections, depending in part on whether the administration is brief or chronic.

In another example, the VEGF-specific antagonist compound is administered locally, e.g., by direct injections, when the disorder or location of the tumor permits, and the injections can be repeated periodically. The VEGF-specific antagonist can also be delivered systemically to the subject or directly to the tumor cells, e.g., to a tumor or a tumor bed following surgical excision of the tumor, in order to prevent or reduce local recurrence or metastasis, for example of a dormant tumor or micrometastases.

Alternatively, an inhibitory nucleic acid molecule or polynucleotide containing a nucleic acid sequence encoding a VEGF-specific antagonist can be delivered to the appropriate cells in the subject. In certain embodiments, the nucleic acid can be directed to the tumor itself.

The nucleic acid can be introduced into the cells by any means appropriate for the vector employed. Many such methods are well known in the art (Sambrook et al., *supra*, and Watson et al., Recombinant DNA, Chapter 12, 2d edition, Scientific American Books, 1992).

Examples of methods of gene delivery include liposome mediated transfection, electroporation, calcium phosphate/DEAE dextran methods, gene gun, and microinjection.

V. PHARMACEUTICAL FORMULATIONS

Therapeutic formulations of the agents (e.g., antibodies) used in accordance with the invention are prepared for storage by mixing an antibody having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (*e.g.* Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG). Lyophilized anti-VEGF antibody formulations are described in WO 97/04801, expressly incorporated herein be reference.

Optionally, but preferably, the formulation contains a pharmaceutically acceptable salt, typically, e.g., sodium chloride, and preferably at about physiological concentrations. Optionally, the formulations of the invention can contain a pharmaceutically acceptable preservative. In some embodiments the preservative concentration ranges from 0.1 to 2.0%, typically v/v. Suitable preservatives include those known in the pharmaceutical arts. Benzyl alcohol, phenol, m-cresol, methylparaben, and propylparaben are examples of preservatives. Optionally, the formulations of the invention can include a pharmaceutically acceptable surfactant at a concentration of 0.005 to 0.02%.

Typically, bevacizumab is supplied for therapeutic uses in 100 mg and 400 mg preservative-free, single-use vials to deliver 4 ml or 16 ml of bevacizumab (25 mg/ml). The 100 mg product is formulated in 240 mg α, α-trehalose dehydrate, 23.2 mg sodium phosphate

(monobasic, monohydrate), 4.8 mg sodium phosphate (dibasic, anhydrous), 1.6 mg polysorbate 20, and Water for Injection, USP. The 400 mg product is formulated in 960 mg α, α-trehalose dehydrate, 92.8 mg sodium phosphate (monobasic, monohydrate), 19.2 mg sodium phosphate (dibasic, anhydrous), 6.4 mg polysorbate 20, and Water for Injection, USP. See also the label for bevacizumab.

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. For example, it may be desirable to further provide antibodies which bind to EGFR, VEGF (*e.g.* an antibody which binds a different epitope on VEGF), VEGFR, or ErbB2 (e.g., Herceptin®) in the one formulation. Alternatively, or in addition, the composition may comprise a cytotoxic agent, cytokine, growth inhibitory agent and/or VEGFR antagonist. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980).

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g., films, or microcapsule. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C, resulting in a loss of biological activity and possible changes in

immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

The formulations to be used for *in vivo* administration may be sterile. This is readily accomplished by filtration through sterile filtration membranes.

VI. EFFICACY OF THE TREATMENT

The main advantage of the of any of the methods, uses and compositions provided herein is the ability of producing marked anti-cancer effects in a human subject without causing significant toxicities or adverse effects, so that the subject benefited from the treatment overall. In one embodiment of any of the methods, uses or compositions, the safety profile is comparable to previous bevacizumab phase III studies. The efficacy of the treatment of the invention can be measured by various endpoints commonly used in evaluating cancer treatments, including but not limited to, tumor regression, tumor weight or size shrinkage, time to progression, duration of survival, progression free survival, overall response rate, duration of response, and quality of life. Because the anti-angiogenic agents of the invention target the tumor vasculature and not necessarily the neoplastic cells themselves, they represent a unique class of anticancer drugs, and therefore may require unique measures and definitions of clinical responses to drugs. For example, tumor shrinkage of greater than 50% in a 2-dimensional analysis is the standard cut-off for declaring a response. However, the anti-VEGF antibody of the invention may cause inhibition of metastatic spread without shrinkage of the primary tumor, or may simply exert a tumouristatic effect. Accordingly, novel approaches to determining efficacy of an anti-angiogenic therapy should be employed, including for example, measurement of plasma or urinary markers of angiogenesis and measurement of response through radiological imaging.

In another embodiment, the invention provides methods for increasing progression free survival of a human subject susceptible to or diagnosed with a cancer. Time to disease progression is defined as the time from administration of the drug until disease progression or death. In a preferred embodiment, the combination treatment of the invention using anti-VEGF antibody and one or more chemotherapeutic agents significantly increases progression free survival by at least about 1 month, 1.2 months, 2 months, 2.4 months, 2.9 months, 3.5 months, preferably by about 1 to about 5 months, when compared to a treatment with

chemotherapy alone. In one embodiment, the PFS median in months (95% CI) is 9.2 months (8.6, 10.1) in the subjects treated with bevacizumab and taxane therapy (e.g., docetaxel or paclitaxel protein-bound particles (e.g., Abraxane®))/anthracycline therapy (e.g., doxorubicin, epirubicin or combinations thereof) compared to 8.0 months (6.7, 8.4) the taxane/anthracycline therapy without bevacizumab, with a HR (95% CI) 0.644 (0.522, 0.795), p-value (log-rank) less than 0.0001. In one embodiment, the PFS in the subjects treated with bevacizumab and taxane/anthracycline is 10.7 months compared to 8.3 in subjects treated with placebo and taxane/anthracycline. In one embodiment, the PFS median in months (95% CI) is 8.6 months (8.1, 9.5) in the subjects treated with bevacizumab and capecitabine compared to 5.7 months (4.3, 6.2) with capecitabine therapy without bevacizumab, with a HR (95% CI) 0.688 (0.564, 0.840), p-value (log-rank) 0.0002. In one embodiment, the PFS in the subjects treated with bevacizumab and capecitabine is 9.7 months compared to 6.2 in subjects treated with placebo and capecitabine.

In yet another embodiment, the treatment of the invention significantly increases response rate in a group of human subjects susceptible to or diagnosed with a cancer who are treated with various therapeutics. Response rate is defined as the percentage of treated subjects who responded to the treatment. In one aspect, the combination treatment of the invention using anti-VEGF antibody and one or more chemotherapeutic agents significantly increases response rate in the treated subject group compared to the group treated with chemotherapy alone.

In one aspect, the invention provides methods for increasing duration of response in a human subject or a group of human subjects susceptible to or diagnosed with a cancer. Duration of response is defined as the time from the initial response to disease progression.

In one embodiment, the invention can be used for increasing the duration of survival of a human subject susceptible to or diagnosed with a cancer.

VII. Antibody Production

(i) Polyclonal antibodies

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, *e.g.*, keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-

hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N=C=NR$, where R and R^1 are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, *e.g.*, 100 μg or 5 μg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

(ii) Monoclonal antibodies

Various methods for making monoclonal antibodies herein are available in the art. For example, the monoclonal antibodies may be made using the hybridoma method first described by Kohler *et al., Nature,* 256:495 (1975), or by recombinant DNA methods (U.S. Patent No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster or macaque monkey, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized *in vitro*. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, California USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Maryland USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.*, 133:3001 (1984); Brodeur *et al., Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown *in vivo* as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the monoclonal antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the

recombinant host cells. Recombinant production of antibodies will be described in more detail below.

In a further embodiment, antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty *et al., Nature*, 348:552-554 (1990). Clackson *et al., Nature*, 352:624-628 (1991) and Marks *et al., J. Mol. Biol.*, 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks *et al., Bio/Technology*, 10:779-783 (1992)), as well as combinatorial infection and *in vivo* recombination as a strategy for constructing very large phage libraries (Waterhouse *et al., Nuc. Acids. Res.*, 21:2265-2266 (1993)). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Patent No. 4,816,567; Morrison, *et al., Proc. Natl Acad. Sci. USA*, 81:6851 (1984)), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

(iii) Humanized and human antibodies

A humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones *et al., Nature*, 321:522-525 (1986); Riechmann *et al., Nature*, 332:323-327 (1988); Verhoeyen *et al., Science*, 239:1534-1536 (1988)), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Patent No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which

some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody (Sims *et al., J. Immunol.*, 151:2296 (1993); Chothia *et al., J. Mol. Biol.*, 196:901 (1987)). Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter *et al., Proc. Natl. Acad. Sci. USA*, 89:4285 (1992); Presta *et al., J. Immnol.*, 151:2623 (1993)).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, *i.e.*, the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

Humanized anti-VEGF antibodies and affinity matured variants thereof are described in, for example, U.S. Pat. No. 6,884,879 issued February 26, 2005.

It is now possible to produce transgenic animals (*e.g.*, mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production.

Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, *e.g.*, Jakobovits *et al., Proc. Natl. Acad. Sci. USA*, 90:2551 (1993); Jakobovits *et al., Nature*, 362:255-258 (1993); Bruggermann *et al., Year in Immuno.*, 7:33 (1993); and Duchosal *et al.* *Nature* 355:258 (1992).

Alternatively, phage display technology (McCafferty *et al.*, *Nature* 348:552-553 (1990)) can be used to produce human antibodies and antibody fragments *in vitro*, from immunoglobulin variable (V) domain gene repertoires from unimmunized donors. According to this technique, antibody V domain genes are cloned in-frame into either a major or minor coat protein gene of a filamentous bacteriophage, such as M13 or fd, and displayed as functional antibody fragments on the surface of the phage particle. Because the filamentous particle contains a single-stranded DNA copy of the phage genome, selections based on the functional properties of the antibody also result in selection of the gene encoding the antibody exhibiting those properties. Thus, the phage mimics some of the properties of the B-cell. Phage display can be performed in a variety of formats; for their review see, *e.g.*, Johnson, Kevin S. and Chiswell, David J., *Current Opinion in Structural Biology* 3:564-571 (1993). Several sources of V-gene segments can be used for phage display. Clackson *et al.*, *Nature*, 352:624-628 (1991) isolated a diverse array of anti-oxazolone antibodies from a small random combinatorial library of V genes derived from the spleens of immunized mice. A repertoire of V genes from unimmunized human donors can be constructed and antibodies to a diverse array of antigens (including self-antigens) can be isolated essentially following the techniques described by Marks *et al.*, *J. Mol. Biol.* 222:581-597 (1991), or Griffith *et al.*, *EMBO J.* 12:725-734 (1993). See, also, U.S. Patent Nos. 5,565,332 and 5,573,905.

As discussed above, human antibodies may also be generated by *in vitro* activated B cells (see U.S. Patents 5,567,610 and 5,229,275).

Human monoclonal anti-VEGF antibodies are described in U.S. Patent No. 5,730,977, issued March 24, 1998.

(iv) Antibody fragments

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, *e.g.*, Morimoto *et al.* , *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992) and Brennan *et al., Science*, 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from

the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter *et al.*, Bio/Technology 10:163-167 (1992)). According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185.

(v) Other amino acid sequence modifications

Amino acid sequence modification(s) of the antibodies described herein are contemplated. For example, it may be desirable to improve the binding affinity and/or other biological properties of the antibody. Amino acid sequence variants of the antibody are prepared by introducing appropriate nucleotide changes into the antibody nucleic acid, or by peptide synthesis. Such modifications include, for example, deletions from, and/or insertions into and/or substitutions of, residues within the amino acid sequences of the antibody. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid changes also may alter post-translational processes of the antibody, such as changing the number or position of glycosylation sites.

A useful method for identification of certain residues or regions of the antibody that are preferred locations for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells *Science*, 244:1081-1085 (1989). Here, a residue or group of target residues are identified (*e.g.*, charged residues such as arg, asp, his, lys, and glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with antigen. Those amino acid locations demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants at, or for, the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation *per se* need not be predetermined. For example, to analyze the performance of a mutation at a given site, ala scanning or random mutagenesis is conducted at the target codon or region and the expressed antibody variants are screened for the desired activity.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of terminal insertions include antibody with an N-terminal methionyl residue or the antibody fused to a cytotoxic polypeptide. Other insertional variants of the antibody molecule include

the fusion to the N- or C-terminus of the antibody to an enzyme (*e.g.* for ADEPT) or a polypeptide which increases the serum half-life of the antibody.

Another type of variant is an amino acid substitution variant. These variants have at least one amino acid residue in the antibody molecule replaced by a different residue. The sites of greatest interest for substitutional mutagenesis include the hypervariable regions, but FR alterations are also contemplated.

Substantial modifications in the biological properties of the antibody are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Amino acids may be grouped according to similarities in the properties of their side chains (in A. L. Lehninger, in *Biochemistry*, second ed., pp. 73-75, Worth Publishers, New York (1975)):

(1) non-polar: Ala (A), Val (V), Leu (L), Ile (I), Pro (P), Phe (F), Trp (W), Met (M)

(2) uncharged polar: Gly (G), Ser (S), Thr (T), Cys (C), Tyr (Y), Asn (N), Gln (Q)

(3) acidic: Asp (D), Glu (E)

(4) basic: Lys (K), Arg (R), His(H)

Alternatively, naturally occurring residues may be divided into groups based on common side-chain properties:

(1) hydrophobic: Norleucine, Met, Ala, Val, Leu, Ile;

(2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln;

(3) acidic: Asp, Glu;

(4) basic: His, Lys, Arg;

(5) residues that influence chain orientation: Gly, Pro;

(6) aromatic: Trp, Tyr, Phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another class.

Any cysteine residue not involved in maintaining the proper conformation of the antibody also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking. Conversely, cysteine bond(s) may be added

to the antibody to improve its stability (particularly where the antibody is an antibody fragment such as an Fv fragment).

A particularly preferred type of substitutional variant involves substituting one or more hypervariable region residues of a parent antibody (*e.g.* a humanized or human antibody). Generally, the resulting variant(s) selected for further development will have improved biological properties relative to the parent antibody from which they are generated. A convenient way for generating such substitutional variants involves affinity maturation using phage display. Briefly, several hypervariable region sites (*e.g.* 6-7 sites) are mutated to generate all possible amino substitutions at each site. The antibody variants thus generated are displayed in a monovalent fashion from filamentous phage particles as fusions to the gene III product of M13 packaged within each particle. The phage-displayed variants are then screened for their biological activity (*e.g.* binding affinity) as herein disclosed. In order to identify candidate hypervariable region sites for modification, alanine scanning mutagenesis can be performed to identify hypervariable region residues contributing significantly to antigen binding. Alternatively, or additionally, it may be beneficial to analyze a crystal structure of the antigen-antibody complex to identify contact points between the antibody and human VEGF. Such contact residues and neighboring residues are candidates for substitution according to the techniques elaborated herein. Once such variants are generated, the panel of variants is subjected to screening as described herein and antibodies with superior properties in one or more relevant assays may be selected for further development.

Another type of amino acid variant of the antibody alters the original glycosylation pattern of the antibody. By altering is meant deleting one or more carbohydrate moieties found in the antibody, and/or adding one or more glycosylation sites that are not present in the antibody.

Glycosylation of antibodies is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-aceylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to the antibody is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the sequence of the original antibody (for O-linked glycosylation sites).

Where the antibody comprises an Fc region, the carbohydrate attached thereto may be altered. For example, antibodies with a mature carbohydrate structure that lacks fucose attached to an Fc region of the antibody are described in US Pat Appl No US 2003/0157108 A1, Presta, L. See also US 2004/0093621 A1 (Kyowa Hakko Kogyo Co., Ltd). Antibodies with a bisecting N-acetylglucosamine (GlcNAc) in the carbohydrate attached to an Fc region of the antibody are referenced in WO03/011878, Jean-Mairet *et al.* and US Patent No. 6,602,684, Umana *et al.* Antibodies with at least one galactose residue in the oligosaccharide attached to an Fc region of the antibody are reported in WO97/30087, Patel *et al.* See, also, WO98/58964 (Raju, S.) and WO99/22764 (Raju, S.) concerning antibodies with altered carbohydrate attached to the Fc region thereof.

It may be desirable to modify the antibody of the invention with respect to effector function, *e.g.* so as to enhance antigen-dependent cell-mediated cytotoxicity (ADCC) and/or complement dependent cytotoxicity (CDC) of the antibody. This may be achieved by introducing one or more amino acid substitutions in an Fc region of the antibody. Alternatively or additionally, cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). See Caron *et al.*, *J. Exp Med.* 176:1191-1195 (1992) and Shopes, B. *J. Immunol.* 148:2918-2922 (1992). Homodimeric antibodies with enhanced anti-tumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff *et al. Cancer Research* 53:2560-2565 (1993). Alternatively, an antibody can be engineered which has dual Fc regions and may thereby have enhanced complement lysis and ADCC capabilities. See Stevenson *et al. Anti-Cancer Drug Design* 3:219-230 (1989).

WO00/42072 (Presta, L.) describes antibodies with improved ADCC function in the presence of human effector cells, where the antibodies comprise amino acid substitutions in the Fc region thereof. Preferably, the antibody with improved ADCC comprises substitutions at positions 298, 333, and/or 334 of the Fc region (Eu numbering of residues). Preferably the

altered Fc region is a human IgG1 Fc region comprising or consisting of substitutions at one, two or three of these positions. Such substitutions are optionally combined with substitution(s) which increase C1q binding and/or CDC.

Antibodies with altered C1q binding and/or complement dependent cytotoxicity (CDC) are described in WO99/51642, US Patent No. 6,194,551B1, US Patent No. 6,242,195B1, US Patent No. 6,528,624B1 and US Patent No. 6,538,124 (Idusogie *et al.*). The antibodies comprise an amino acid substitution at one or more of amino acid positions 270, 322, 326, 327, 329, 313, 333 and/or 334 of the Fc region thereof (Eu numbering of residues).

To increase the serum half life of the antibody, one may incorporate a salvage receptor binding epitope into the antibody (especially an antibody fragment) as described in US Patent 5,739,277, for example. As used herein, the term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (*e.g.,* IgG_1, IgG_2, IgG_3, or IgG_4) that is responsible for increasing the *in vivo* serum half-life of the IgG molecule.

Antibodies with improved binding to the neonatal Fc receptor (FcRn), and increased half-lives, are described in WO00/42072 (Presta, L.) and US2005/0014934A1 (Hinton *et al.*). These antibodies comprise an Fc region with one or more substitutions therein which improve binding of the Fc region to FcRn. For example, the Fc region may have substitutions at one or more of positions 238, 250, 256, 265, 272, 286, 303, 305, 307, 311, 312, 314, 317, 340, 356, 360, 362, 376, 378, 380, 382, 413, 424, 428 or 434 (Eu numbering of residues). The preferred Fc region-comprising antibody variant with improved FcRn binding comprises amino acid substitutions at one, two or three of positions 307, 380 and 434 of the Fc region thereof (Eu numbering of residues). In one embodiment, the antibody has 307/434 mutations.

Engineered antibodies with three or more (preferably four) functional antigen binding sites are also contemplated (US Appln No. US2002/0004587 A1, Miller *et al.*).

Nucleic acid molecules encoding amino acid sequence variants of the antibody are prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of the antibody.

(vi) Immunoconjugates

The invention also pertains to immunoconjugates comprising the antibody described herein conjugated to a cytotoxic agent such as a chemotherapeutic agent, toxin (*e.g.* an enzymatically active toxin of bacterial, fungal, plant or animal origin, or fragments thereof), or a radioactive isotope (*i.e.,* a radioconjugate).

Chemotherapeutic agents useful in the generation of such immunoconjugates have been described above. Enzymatically active toxins and fragments thereof which can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolaca americana* proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. A variety of radionuclides are available for the production of radioconjugate antibodies. Examples include ^{212}Bi, ^{131}I, ^{131}In, ^{90}Y and ^{186}Re.

Conjugates of the antibody and cytotoxic agent are made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutareldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta *et al. Science* 238: 1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO94/11026.

In another embodiment, the antibody may be conjugated to a "receptor" (such streptavidin) for utilization in tumor pretargeting wherein the antibody-receptor conjugate is administered to the subject, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a "ligand" (*e.g.* avidin) which is conjugated to a cytotoxic agent (*e.g.* a radionucleotide).

(vii) Immunoliposomes

The antibody disclosed herein may also be formulated as immunoliposomes. Liposomes containing the antibody are prepared by methods known in the art, such as described in Epstein *et al., Proc. Natl. Acad. Sci. USA*, 82:3688 (1985); Hwang *et al., Proc. Natl Acad. Sci. USA*, 77:4030 (1980); and U.S. Pat. Nos. 4,485,045 and 4,544,545. Liposomes with enhanced circulation time are disclosed in U.S. Patent No. 5,013,556.

Particularly useful liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-derivatized phosphatidylethanolamine (PEG-PE). Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter. Fab' fragments of the antibody of the invention can be conjugated to the liposomes as described in Martin *et al. J. Biol. Chem.* 257: 286-288 (1982) via a disulfide interchange reaction. A chemotherapeutic agent is optionally contained within the liposome. See Gabizon *et al. J. National Cancer Inst.*81(19)1484 (1989)

VIII. ARTICLES OF MANUFACTURE AND KITS

In another embodiment of the invention, an article of manufacture containing materials useful for the treatment of the disorders described above is provided. The article of manufacture comprises a container, a label and a package insert. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is effective for treating the condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is an anti-VEGF antibody. The label on, or associated with, the container indicates that the composition is used for treating the condition of choice. The article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes. In addition, the article of manufacture comprises a package inserts with instructions for use, including for example instructing the user of the composition to administer the anti-VEGF antibody composition and a chemotherapeutic agent to the subject, e.g., capecitabine, taxane, anthracycline, paclitaxel, docetaxel, paclitaxel protein-bound particles (e.g., Abraxane®), doxorubicin, epirubicin, 5-fluorouracil, cyclophosphamide or combinations

thereof. The package insert may optionally contain some or all of the results found in Example 1.

The VEGF-specific antagonist can be packaged alone or in combination with other anti-cancer therapeutic compounds as a kit. The kit can include optional components that aid in the administration of the unit dose to subjects, such as vials for reconstituting powder forms, syringes for injection, customized IV delivery systems, inhalers, etc. Additionally, the unit dose kit can contain instructions for preparation and administration of the compositions. In certain embodiments, the instructions comprises instructions for use, including for example instructing the user of the composition to administer the anti-VEGF antibody composition and a chemotherapeutic agent to the subject, e.g., capecitabine, taxane, anthracycline, paclitaxel, docetaxel, paclitaxel protein-bound particles (e.g., Abraxane®), doxorubicin, epirubicin, 5-fluorouracil, cyclophosphamide or combinations thereof. The instructions may optionally contain some or all of the results found in Example 1. The kit may be manufactured as a single use unit dose for one subject, multiple uses for a particular subject (at a constant dose or in which the individual compounds may vary in potency as therapy progresses); or the kit may contain multiple doses suitable for administration to multiple subjects ("bulk packaging"). The kit components may be assembled in cartons, blister packs, bottles, tubes, and the like.

Deposit of Materials

The following hybridoma cell line has been deposited under the provisions of the Budapest Treaty with the American Type Culture Collection (ATCC), Manassas, VA, USA:

Antibody Designation	ATCC No.	Deposit Date
A4.6.1	ATCC HB-10709	March 29, 1991

The following example is intended merely to illustrate the practice of the invention and is not provided by way of limitation. The disclosures of all patent and scientific literatures cited herein are expressly incorporated in their entirety by reference.

EXAMPLE

Example 1. Bevacizumab in Combination with Chemotherapy Regimens in Subjects with Previously Untreated Metastic Breast Cancer

Metastatic breast cancer (MBC) is an incurable disease, with the majority of patients succumbing to their disease within 2 year of diagnosis (Greenberg, et al., 1996, J. Clin. Oncol. 14:2197-205; Dawood, et al., 2008, J. Clin. Oncol. 26:4891-8; and Chia et al., Cancer, 2007, 110:973-9). Of the patients presenting with MBC, approximately 60% will have previously presented with localized disease that has recurred; approximately 40% of patients will present with metastatic disease de novo.

Two prior randomized Phase III trials in MBC have demonstrated benefit from addition of bevacizumab to initial chemotherapy with taxanes. In the pivotal Phase III E2100 trial, progression-free survival (PFS) was significantly longer in patients treated with weekly paclitaxel+ bevacizumab than in those treated with paclitaxel alone (Miller at lea., N. Engl J. Med., 2007, 357:2666-76). Similarly, the AVADO trial, which investigated the combination of bevacizumab (at 7.5 and 15 mg/kg q3w) with docetaxel, found that patients treated with docetaxel+ bevacizumab had progression-free survival (PFS) that was longer than in those treated with docetaxel alone (Miles et al., 2008 ASCO Annual Meeting Chicago, IL). Previously, a randomized Phase III trial (AVF2119g) for previously treated MBC that evaluated the combination of bevacizumab with capecitabine demonstrated that overall response rate (ORR) was higher in those treated with capecitabine + bevacizumab than capecitabine alone, but it failed to meet its primary objective of improving PFS (Miller et al., J. Clin. Oncol. 2005, 23:792-9).

This example concerns analysis of results obtained with previously untreated metastatic breast cancer subjects treated in the RIBBON 1 clinical trial using taxanes and non-taxane chemotherapies. The primary objective of the study was to determine the clinical benefit of the addition of bevacizumab to standard chemotherapy regimes for previously untreated metastatic breast cancer, as measured by PFS based on investigator tumor assessment. *See, e.g.,* O'Shaughnessy and Brufsky, (2008), *Clinical Breast Cancer*, 8(4): 370-373. The trial comprised two study groups that evaluated AVASTIN® with different types of chemotherapies in women who had not previously received chemotherapy for their advanced HER2-negative breast cancer. In the first study group, women received either AVASTIN or placebo in combination with taxane or anthracycline-based chemotherapies. In the second study group, women received either AVASTIN or placebo in combination with capecitabine chemotherapy. The analysis of this example was based on information from 1237 patients. These trials evaluated the efficacy of bevacizumab (AVASTIN®) as therapy for patients previously untreated metastic breast cancer.

Study Design

The design of the RIBBON1 study is depicted in **Figure 1**.

In the RIBBON1 trial, the following treatment protocol was used:

Arm A: bevacizumab 15mg/kg IV on day 1 of each 21-day cycle and either cohort 1, cohort 2 or cohort 3;

Arm B: placebo IV on day 1 of each 21-day cycle and either cohort 1, cohort 2 or cohort 3.

Cohort 1: Either of the following taxanes administered every 3 weeks

Docetaxel 75-100 mg/m^2 IV

Paclitaxel protein-bound particles (Abraxane ®) 260 mg/m^2 IV

Cohort 2: Any of the following anthracycline-based combination chemotherapies, for subjects previously untreated with anthracyclines, every 3 weeks:

FEC: 5-fluorouracil 500 mg/m^2 IV, epirubicin 90-100 mg/m^2 IV and cyclophosphamide 500 mg/m^2 IV on Day 1

FAC: 5-fluorouracil 500 mg/m^2 IV, doxorubicin 50 mg/m^2 IV and cyclophosphamide 500 mg/m^2 IV on Day 1

AC: Doxorubicin 50-60 mg/m^2 IV and cyclophosphamide 500-600 mg/m^2 IV on Day 1

EC: Epirubicin 90-100 mg/m^2 IV and cyclophosphamide 500-600 mg/m^2 IV on Day 1

Cohort 3: Capecitabine 1000 mg/m^2 oral twice daily on Days 1-14 of each 3-week cycle.

In addition, after the blinded treatment phase, some subjects were given bevacizumab either 15 mg/kg IV every three weeks or 10 mg/ml IV every 2 weeks; given concurrently with chemotherapy.

Bevacizumab (AVASTIN®) was supplied as a clear to slightly opalescent, colorless to pale brown, sterile liquid concentrate for solution for IV infusion. Bevacizumab was supplied in either a 5-ml (100 mg) or 20-ml (400 mg) glass vials containing 4 mL or 16 mL bevacizumab, respectively (25 mg/ml for either vial). Vials contain bevacizumab with phosphate, trehalose, polysorbate 20, and Sterile Water for Injection (SWFI), USP. Vials contained no preservative. AVASTIN® was diluted in 0.9% Sodium Chloride Injection, USP, to a total volume of 100 ml before continuous intravenous administration.

Methods

Eligible Subjects/Patients had the following key eligibility criteria: Age > 18 years, ECOG 0 or 1 (ECOG Performance Status Scale), no prior chemotherapy for locally recurrent

or metastatic breast cancer, Her2 negative (unless Her2 positive and trastuzumab contraindicated or unavailable) and/or prior adjuvant chemotherapy allowed if recurrence > (or equal to) 12 months since last dose. All subjects had histologically or cytologically confirmed adenocarcinoma of the breast, subjects may have had either measureable (per the Response Evaluation Criteria in Solid Tumors (RECIST)) or non-measureable locally recurrent or metastatic disease. The locally recurrent disease was not amenable to resection with curative intent.

Subjects may have received prior hormonal therapy in either the adjuvant or metastatic setting if discontinued greater than or equal to 1 week prior to Day 0, or adjuvant chemotherapy if discontinued greater than or equal to 12 months prior to Day 0.

Exclusion criteria included known HER2-positive status (unless the patient had progressed on trastuzumab therapy or trastuzumab therapy was contraindicated or unavailable); prior adjuvant or neo adjuvant chemotherapy within 12 months; known central nervous system metastases; blood pressure >150/100 mmHg; unstable angina; New York Heart Association Grade II or greater congestive heart failure (CHF); history of myocardial infarction within 6 months; history of stroke or transient ischemic attack within 6 months; clinically significant peripheral vascular disease; evidence of bleeding diathesis or coagulopathy; history of abdominal fistula, gastrointestinal (GI) perforation, or intra abdominal abscess within 6 months; history of anaphylactic reaction to monoclonal antibody therapy not controlled with premedication; serious non healing wound; inadequate organ function; locally recurrent disease amenable to resection with curative intent; history of other malignancies within 5 years. If anthracycline chosen as chemotherapy, patients were also required to have left ejection fraction ≥50% and no prior history of anthracycline treatment.

The trial was conducted worldwide (at least 22 countries) and accrued 1237 subjects/patients (Taxane (T): 307; Anthracycline (Anthra): 315; and Capecitabine (Cap): 615).

The primary endpoint of the study was progression free survival (PFS), defined as the time from randomization to disease progression or to death, based on investigator assessment. Kaplan-Meier methodology can be used to estimate median PFS for each treatment arm. In certain embodiments, the hazard ratio for PFS will be estimated using a stratified Cox regression model with the same stratification factors used in the stratified log-rank test. Analyses of PFS in each cohort is performed at the two-sided α=0.05 level. Time-to-event data are compared between treatment arms using a stratified log-rank test. The Kaplan-Meier

method is used to estimate duration of time-to-event data. The 95% confidence intervals for median time-to-event are computed using the Brookmeyer-Crowley method. The HR for time-to-event data are estimated using a stratified Cox regression model.

The secondary endpoints included objective response rate (ORR), one-year survival rate, overall survival (OS), and PFS based on IRC assessment and safety. OS is defined as the time from randomization until death from any cause. ORR is defined as the percentage of patients who achieved a complete or partial response confirmed ≥28 days after initial documentation of response. One-year survival rate is assessed between treatment arms using the normal approximation method. ORR in patients with measurable disease at baseline is compared using the stratified Mantel-Haenszel $\chi 2$ test. Randomization stratification factors are included in all stratified analyses.

Results

RIBBON1 was an international, multicenter, randomized, double-blind, placebo-controlled clinical study that enrolled 1,237 subjects/patients with locally recurrent or metastatic HER2-negative breast cancer who had not received chemotherapy for their metastatic disease. See Table 1 for Subject/Patient Characteristics from the trial. The primary endpoint of these trials was progression free survival (PFS), defined as the time from randomization to disease progression or death, based on investigator assessment. The results from the trial indicate that AVASTIN® in combination with the following used chemotherapies for first-line metastatic HER2-negative breast cancer increased the time women lived without their disease advancing, as defined as the primary endpoint of progression-free survival (PFS), compared to chemotherapies alone.

Table 1: Subject/Patient Characteristics

	Cap		T/Anthra	
	PL (n=206)	BV (n=409)	PL (n=207)	BV (n=415)
Median age, yr	57	56	55	55
ECOG PS 0	53	52	53	52
HR positive	71	76	74	74
Triple negative	24	21	22	23
Disease-free≤12	22	27	41	37

months				
Adjuvant chemotherapy	76	70	47	45
Taxane	41	39	15	15
Anthracycline	69	60	30	30
≥ 3 metastatic sites	45	43	45	45
Measurable dx	78	80	86	83

The results of this phase III study provide direct support for use of antiangiogenic agents as first line therapy for patients with previously untreated breast cancer. The addition of bevacizumab, an anti-VEGF antibody, to the taxane therapy (e.g., docetaxel or paclitaxel protein-bound particles (e.g., Abraxane®))/anthracycline therapy (e.g., doxorubicin, epirubicin or combinations thereof) or capecitabine therapy chemotherapy conferred a clinically meaningful and statistically significant improvement in breast cancer patients as measured by, for example, progression-free survival. The PFS median in months (95% CI) is 9.2 months (8.6, 10.1) in the patients treated with bevacizumab and taxane therapy (e.g., docetaxel or paclitaxel protein-bound particles (e.g., Abraxane®))/anthracycline therapy (e.g., doxorubicin, epirubicin or combinations thereof) compared to 8.0 months (6.7, 8.4) in the taxane/anthracycline therapy without bevacizumab, with a HR (95% CI) 0.644 (0.522, 0.795), p-value (log-rank) less than 0.0001. See Table 2. See **Figure 3** to see investigator (INV) determined PFS values and independent review committee (IRC) determined PFS values. The PFS median in months (95% CI) is 8.6 months (8.1, 9.5) in the patients treated with bevacizumab and capecitabine compared to 5.7 months (4.3, 6.2) in capecitabine therapy without bevacizumab, with a HR (95% CI) 0.688 (0.564, 0.840), p-value (log-rank) 0.0002. See Table 2. See **Figure 2** to see investigator (INV) determined PFS values and independent review committee (IRC) determined PFS values. See Table 3 for secondary endpoints, where the PFS is divided by chemotherapy subgroups. See **Figures 4 and 6** for subgroup analyses of PFS by various cohorts, e.g., capecitabine and T/anthracycline in **Figure 4**, and T/anthracycline in **Figure 6**. See **Figure 5** for objective response rate (ORR) and Table 2. Among responders, median duration of objective response was longer in the bevacizumab arms for both cohorts : Capecitabine cohort, 9.2 months (95% CI: 8.5–10.4) vs. 7.2 months (95% CI: 5.1–9.3); and for the taxane / anthracycline cohort, 8.3 months (95% CI: 7.2–10.7) vs. 7.1 months (95% CI: 6.2–8.8). See Table 4 for Overall survival details. There is no

unexpected safety signal. Safety was consistent with results of prior bevacizumab trials. See Table 5 for safety summary. This improvement is clinically meaningful.

Table 2 PFS and OS

	T/Anthr n=622		Cap n=615	
	pl n=207	B n=415	pl n=206	B N=409
PFS (HR, 95% CI)	0.644 (0.522, 0.795)		0.688 (0.564, 0.840)	
p-value (Log-rank)	<0.0001		0.0002	
Median (months)	8.0	9.2	5.7	8.6
ORR (%)	67 (37.9%)	177 (51.3%)	38 (23.6%)	115 (35.4%)
p-value	0.0054		0.0097	
OS (HR, 95% CI)	1.032 (0.774, 1.376)		0.847 (0.631, 1.138)	
p-value (Log-rank)	0.8298		0.2706	
Median (months)	23.8	25.2	21.2	29.0

HR=hazard ratio

Table 3 Secondary Endpoint : PFS by Chemotherapy Subgroups (mPFS=median PFS)

	Taxane		Anthra	
	PL(n=104)	BV(n=203)	PL(n=103)	BV(n=212)
mPFS, mo	8.2	9.2	7.9	9.2
HR (95% CI)	0.75 (0.56–1.01)		0.55 (0.40–0.74)	
p-value	0.0547		<0.0001	

Table 4: Overall Survival

	Cap		T/Anthra	
	PL (n=206)	BV (n=409)	PL (n=207)	BV (n=415)
% of deaths	35	30	35	34
Median OS, mo	21.2	29.0	23.8	25.2

HR (95% CI)	0.85 (0.63–1.14)		1.03 (0.77–1.38)	
p-value	0.27		0.83	
1-yr survival rate (%)	74	81	83	81
p-value	0.076		0.44	

Table 5: Safety Summary

	Cape		Taxane		Anthra	
Event (%)	PL (n=201)	BV (n=404)	PL (n=102)	BV (n=203)	PL (n=100)	BV (n=210)
Selected AEs*	9.0	22.0	22.5	43.8	16.0	28.1
SAEs	18.9	24.3	26.5	41.4	16.0	22.4
AEs leading to study drug (PL or BV) discontinuation	11.9	11.9	7.8	24.1	4.0	14.3
AEs leading to death**	2.5	2.0	2.9	3.4	3.0	1.4

* Adverse Events (AEs) previously shown to be associated with bevacizumab

**Excludes AEs related to metastatic breast cancer progression

SAE—severe adverse events

The addition of bevacizumab to capecitabine, taxane or anthracycline-based chemotherapy regimens used in 1st-line treatment of metastatic breast cancer, resulted in statistically-significant improvement in PFS with a safety profile comparable to prior Phase III studies.

WHAT IS CLAIMED IS:

1. A method of treating a subject diagnosed with locally recurrent or metastatic breast cancer, comprising administering to the subject a treatment regimen comprising an effective amount of at least one chemotherapy and an anti-VEGF antibody, wherein said subject has not received any chemotherapy for locally recurrent or metastatic breast cancer, and/or has not received prior adjuvant chemotherapy in recurrence less than or equal to 12 months since last dose, and wherein the treatment regimen effectively extends the progression free survival of the subject.

2. The method of claim 1, wherein the chemotherapeutic agent is capecitabine, taxane, anthracycline, paclitaxel, docetaxel, paclitaxel protein-bound particles (e.g., Abraxane®), doxorubicin, epirubicin, 5-fluorouracil, cyclophosphamide or combinations thereof.

3. The method of claim 1, wherein the chemotherapy of the treatment regimen comprises administration of FEC: 5-fluorouracil, epirubicin, and cyclophosphamide, or FAC: 5-fluorouracil, doxorubicin and cyclophosphamide, or AC: doxorubicin and cyclophosphamide, or EC: Epirubicin and cyclophosphamide.

4. The method of claim 1, wherein said anti-VEGF antibody binds the same epitope as the monoclonal anti-VEGF antibody A4.6.1 produced by hybridoma ATCC HB 10709.

5. The method of claim 1, wherein the anti-VEGF antibody is a humanized antibody.

6. The method of claim 1, wherein the subject is HER2 negative.

7. The method of claim 1, wherein the anti-VEGF antibody is bevacizumab.

8. The method of claim 1, wherein the anti-VEGF antibody is bevacizumab and the chemotherapy is capecitabine.

9. The method of claim 8, wherein the administration of capecitabine is 1000 mg/m2 oral twice daily on Days 1-14 of each 3-week cycle and the administration of bevacizumab is 15mg/kg IV on day 1 of each 21-day cycle.

10. The method of claim 7, wherein the administration of bevacizumab is 15mg/kg IV on day 1 of each 21-day cycle, and the chemotherapy is docetaxel which is administered 75-100 mg/m2 IV or paclitaxel protein-bound particles (Abraxane ®) which is administered 260 mg/m2 IV every 3 weeks, or FEC: 5-fluorouracil which is administered 500 mg/m2 IV, epirubicin which is administered 90-100 mg/m2 IV and cyclophosphamide which is

administered 500 mg/m2 IV on Day 1, or FAC: 5-fluorouracil which is administered 500 mg/m2 IV, doxorubicin which is administered 50 mg/m2 IV and cyclophosphamide which is administered 500 mg/m2 IV on Day 1, or AC: Doxorubicin which is administered 50-60 mg/m2 IV and cyclophosphamide which is administered 500-600 mg/m2 IV on Day 1 or EC: Epirubicin which is administered 90-100 mg/m2 IV and cyclophosphamide which is administered 500-600 mg/m2 IV on Day 1 every three weeks.

11. The method of claim 1, wherein the progression free survival of the subject is extended by at least about 1 month or more when compared to another subject treated with the chemotherapy alone.

12. The method of claim 1, wherein the progression free survival of the subject is extended by at least about 2.9 months when compared to another subject treated with the chemotherapy alone.

13. The method of claim 1, wherein the anti-VEGF antibody has a heavy chain variable region comprising the following amino acid sequence:

EVQLVESGGG LVQPGGSLRL SCAASGYTFT NYGMNWVRQA PGKGLEWVGW

INTYTGEPTY AADFKRRFTF SLDTSKSTAY LQMNSLRAED TAVYYCAKYP

HYYGSSHWYF DVWGQGTLVT VSS (SEQ ID NO.1)

and a light chain variable region comprising the following amino acid sequence:

DIQMTQSPSS LSASVGDRVT ITCSASQDIS NYLNWYQQKP GKAPKVLIYF

TSSLHSGVPS RFSGSGSGTD FTLTISSLQP EDFATYYCQQ YSTVPWTFGQ

GTKVEIKR (SEQ ID NO.2).

14. A kit for treating metastatic breast cancer in a human subject comprising a package comprising an anti-VEGF antibody composition and instructions for using the anti-VEGF antibody composition in combination with taxane therapy or anthracycline therapy, wherein the instructions recite that the progression free survival for subjects receiving taxane therapy or anthracycline therapy and bevacizumab is 9.2 months with a hazard ratio of 0.644.

15. A kit for treating metastatic breast cancer in a human subject comprising a package comprising an anti-VEGF antibody composition and instructions for using the anti-VEGF antibody composition in combination with capecitabine therapy, wherein the instructions

recite that the progression free survival for subjects receiving capecitabine therapy and bevacizumab is 8.6 months with a hazard ratio of 0.688.

16. The kit of claim 14 or 15, wherein the anti-VEGF antibody is bevacizumab.

17. The kit of any one of claims 14 or 15, wherein the subject is previously untreated.

18. The kit of claim 14 or 15, wherein the subject is HER2 negative.

19. Use of an anti-VEGF antibody in the manufacture of a medicament for treating locally recurrent or metastatic breast cancer in a subject, wherein said subject has not received any chemotherapy for locally recurrent or metastatic breast cancer, and/or has not received prior adjuvant chemotherapy in recurrence less than or equal to 12 months since last dose, wherein the use effectively extends the progression free survival of the subject, and wherein the medicament further comprises at least one chemotherapeutic agent.

20. The use of claim 19, wherein the chemotherapeutic agent is capecitabine, taxane, anthracycline, paclitaxel, docetaxel, paclitaxel protein-bound particles (e.g., Abraxane®), doxorubicin, epirubicin, 5-fluorouracil, cyclophosphamide or combinations thereof.

21. The use of claim 19, wherein the chemotherapeutic agent is FEC: 5-fluorouracil, epirubicin, and cyclophosphamide, or FAC: 5-fluorouracil, doxorubicin and cyclophosphamide, or AC: doxorubicin and cyclophosphamide, or EC: Epirubicin and cyclophosphamide.

22. The use of claim 19, wherein the anti-VEGF antibody is a humanized antibody.

23. The use of claim 19, wherein the anti-VEGF antibody is bevacizumab.

24. The use of claim 19, wherein the subject is HER2 negative.

25. The use of claim 19, wherein the anti-VEGF antibody is bevacizumab and the chemotherapeutic agent is capecitabine.

26. The use of claim 25, wherein capecitabine is administered 1000 mg/m2 oral twice daily on Days 1-14 of each 3-week cycle and bevacizumab is administered15mg/kg IV on day 1 of each 21-day cycle.

27. The use of claim 23, wherein the administration of bevacizumab is 15mg/kg IV on day 1 of each 21-day cycle, and the chemotherapy is docetaxel which is administered 75-100 mg/m2 IV or paclitaxel protein-bound particles (Abraxane ®) which is administered 260

mg/m2 IV every 3 weeks, or FEC: 5-fluorouracil which is administered 500 mg/m2 IV, epirubicin which is administered 90-100 mg/m2 IV and cyclophosphamide which is administered 500 mg/m2 IV on Day 1, or FAC: 5-fluorouracil which is administered 500 mg/m2 IV, doxorubicin which is administered 50 mg/m2 IV and cyclophosphamide which is administered 500 mg/m2 IV on Day 1, or AC: Doxorubicin which is administered 50-60 mg/m2 IV and cyclophosphamide which is administered 500-600 mg/m2 IV on Day 1 or EC: Epirubicin which is administered 90-100 mg/m2 IV and cyclophosphamide which is administered 500-600 mg/m2 IV on Day 1 every three weeks.

28. The use of claim 19, wherein the progression free survival of the subject is extended by at least about 1 month or more when compared to another subject treated with the chemotherapy alone.

29. The use of claim 19, wherein the progression free survival of the subject is extended by at least about 2.9 months when compared to another subject treated with the chemotherapy alone.

30. An anti-VEGF antibody for use in a method of treating locally recurrent or metastatic breast cancer in a subject, the method comprising administering to the subject a treatment regimen comprising an effective amount of at least one chemotherapy and an anti-VEGF antibody, wherein said subject has not received any chemotherapy for locally recurrent or metastatic breast cancer, and/or has not received prior adjuvant chemotherapy in recurrence less than or equal to 12 months since last dose, and wherein the treatment regimen effectively extends the progression free survival of the subject.

31. The anti-VEGF antibody of claim 30, wherein the chemotherapeutic agent is capecitabine, taxane, anthracycline, paclitaxel, docetaxel, paclitaxel protein-bound particles (e.g., Abraxane®), doxorubicin, epirubicin, 5-fluorouracil, cyclophosphamide or combinations thereof.

32. The anti-VEGF antibody of claim 30, wherein the chemotherapy of the treatment regimen comprises administration of FEC: 5-fluorouracil, epirubicin, and cyclophosphamide, or FAC: 5-fluorouracil, doxorubicin and cyclophosphamide, or AC: doxorubicin and cyclophosphamide, or EC: Epirubicin and cyclophosphamide.

33. The anti-VEGF antibody of claim 30, wherein the anti-VEGF antibody is a humanized antibody.

34. The anti-VEGF antibody of claim 30, wherein the anti-VEGF antibody is bevacizumab.

35. The anti-VEGF antibody of claim 30, wherein the subject is HER2 negative.

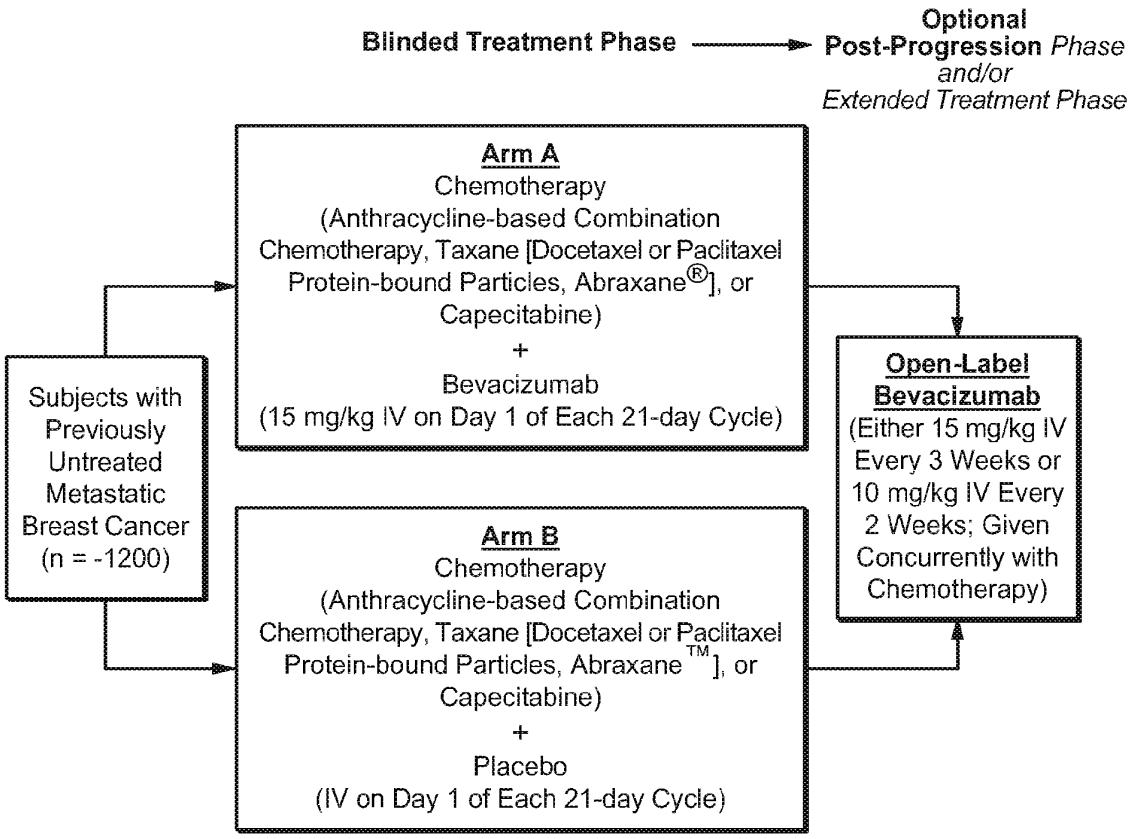
36. The anti-VEGF antibody of claim 30, wherein the anti-VEGF antibody is bevacizumab and the chemotherapy is capecitabine.

37. The anti-VEGF antibody of claim 36, wherein the administration of capecitabine is 1000 mg/m2 oral twice daily on Days 1-14 of each 3-week cycle and the administration of bevacizumab is 15mg/kg IV on day 1 of each 21-day cycle.

38. The anti-VEGF antibody of claim 34, wherein the administration of bevacizumab is 15mg/kg IV on day 1 of each 21-day cycle, and the chemotherapy is docetaxel which is administered 75-100 mg/m2 IV or paclitaxel protein-bound particles (Abraxane ®) which is administered 260 mg/m2 IV every 3 weeks, or FEC: 5-fluorouracil which is administered 500 mg/m2 IV, epirubicin which is administered 90-100 mg/m2 IV and cyclophosphamide which is administered 500 mg/m2 IV on Day 1, or FAC: 5-fluorouracil which is administered 500 mg/m2 IV, doxorubicin which is administered 50 mg/m2 IV and cyclophosphamide which is administered 500 mg/m2 IV on Day 1, or AC: Doxorubicin which is administered 50-60 mg/m2 IV and cyclophosphamide which is administered 500-600 mg/m2 IV on Day 1 or EC: Epirubicin which is administered 90-100 mg/m2 IV and cyclophosphamide which is administered 500-600 mg/m2 IV on Day 1 every three weeks.

39. The anti-VEGF antibody of claim 30, wherein the progression free survival of the subject is extended by at least about 1 month or more when compared to another subject treated with the chemotherapy alone.

40. The anti-VEGF antibody of claim 30, wherein the progression free survival of the subject is extended by at least about 2.9 months when compared to another subject treated with the chemotherapy alone.



Cohort 1: Either of the following taxanes administered every 3 weeks:
Docetaxel 75-100 mg/m^2 IV
Paclitaxel protein-bound particles (Abraxane®) 260 mg/m^2 IV
Cohort 2: Any of the following anthracycline-based combination chemotherapies, for subjects previously untreated with anthracyclines, every 3 weeks:
FEC: 5-fluorouracil 500 mg/m^2 IV, epirubicin 90-100 mg/m^2 IV, and cyclophosphamide 500 mg/m^2 IV on Day 1
FAC: 5-fluorouracil 500 mg/m^2 IV, doxorubicin 50 mg/m^2 IV, and cyclophosphamide 500 mg/m^2 IV on Day 1
AC: Doxorubicin 50-60 mg/m^2 IV and cyclophosphamide 500-600 mg/m^2 IV on Day 1
EC: Epirubicin 90-100 mg/m^2 IV and cyclophosphamide 500-600 mg/m^2 IV on Day 1
Cohort 3: Capecitabine 1000 mg/m^2 oral twice daily on Days 1-14 of each 3-week cycle

FIG. 1

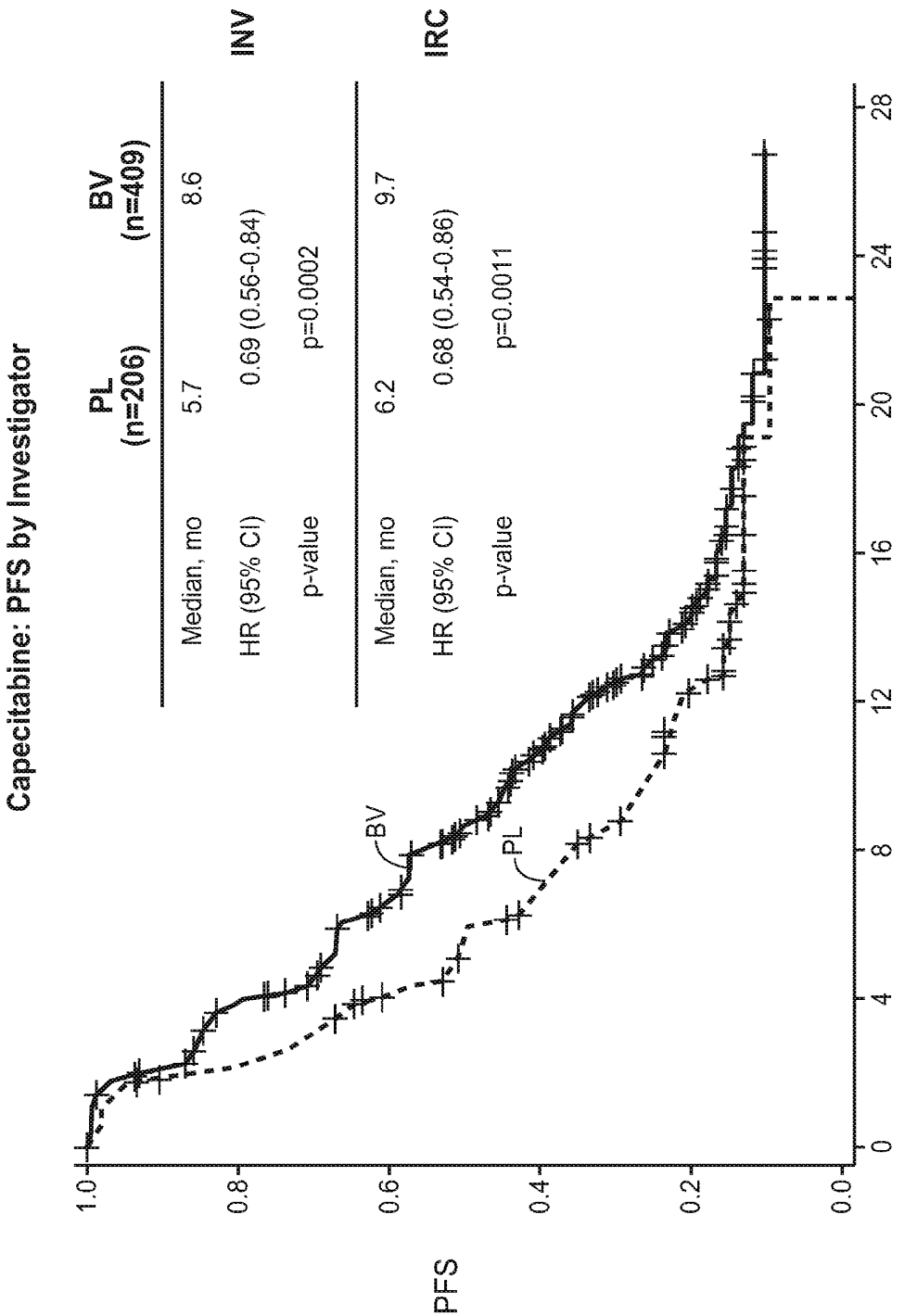


FIG. 2

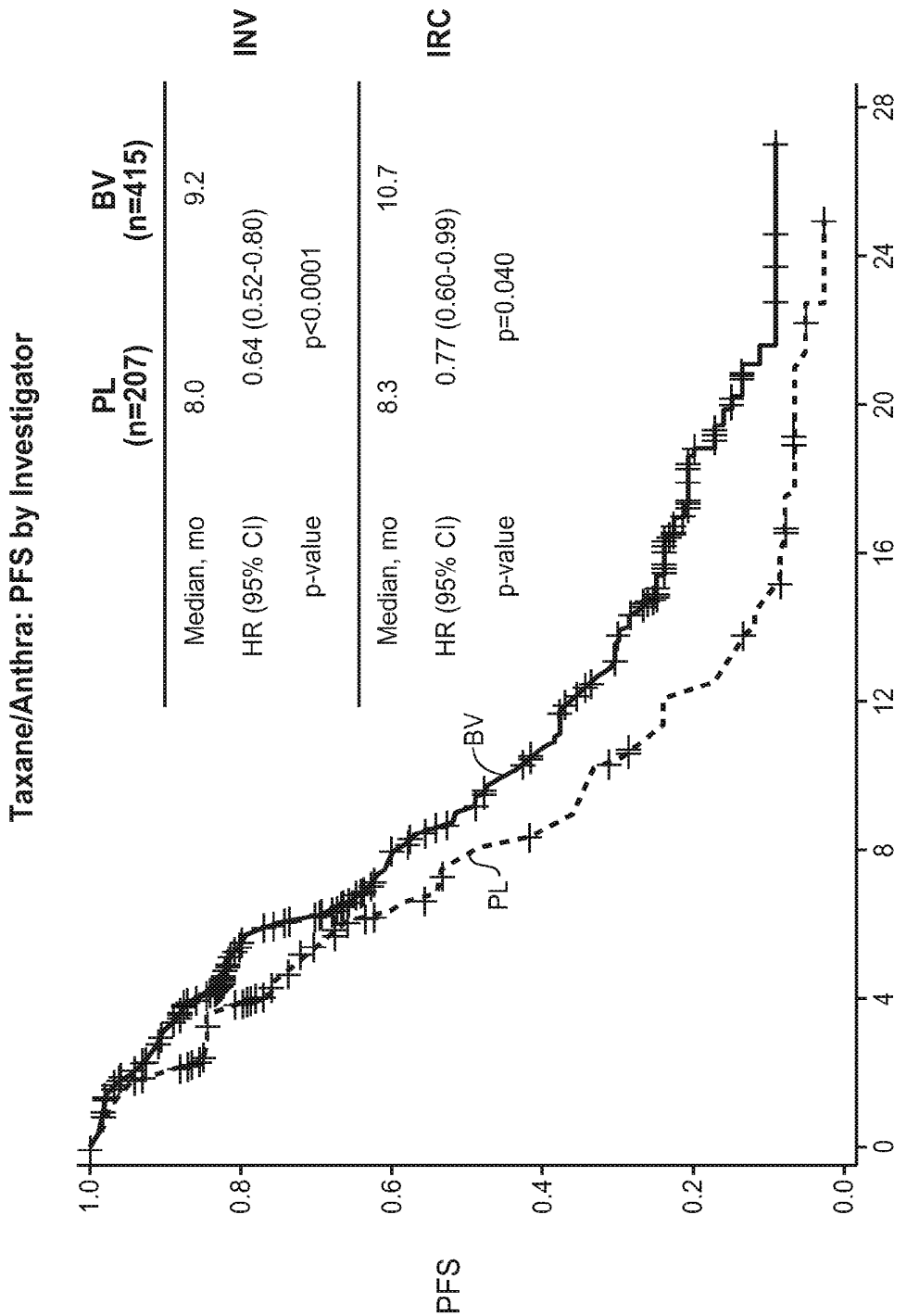


FIG. 3

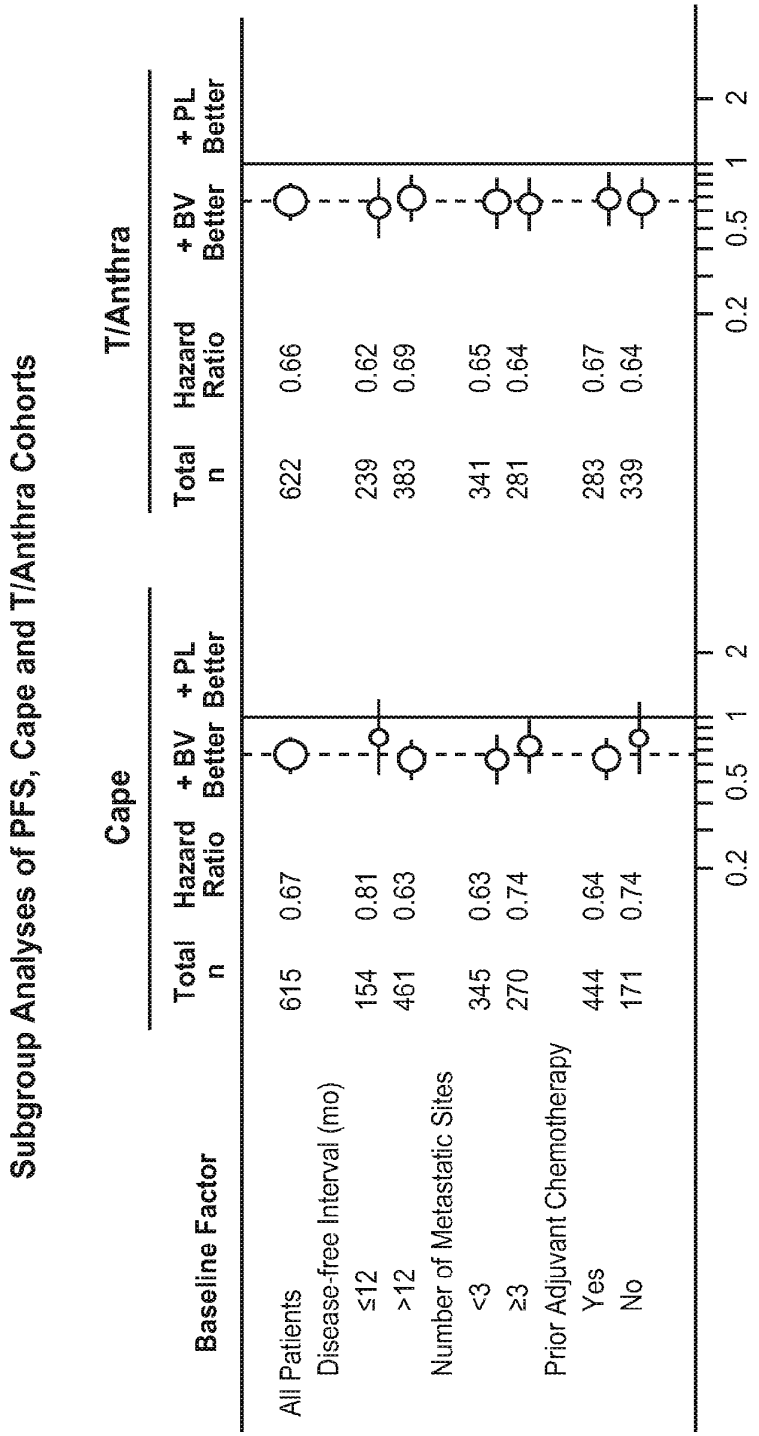


FIG. 4

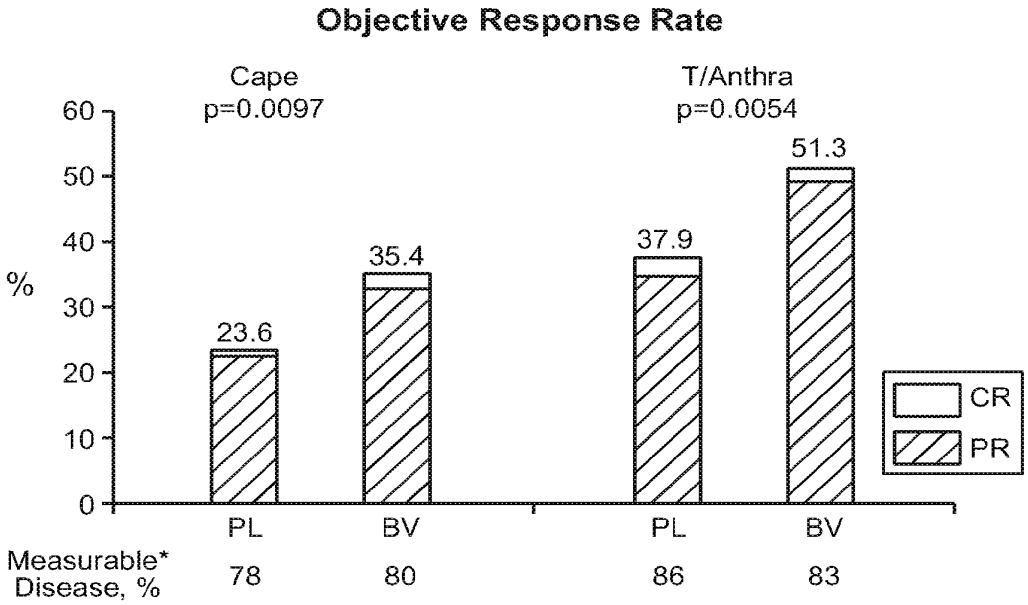


FIG. 5

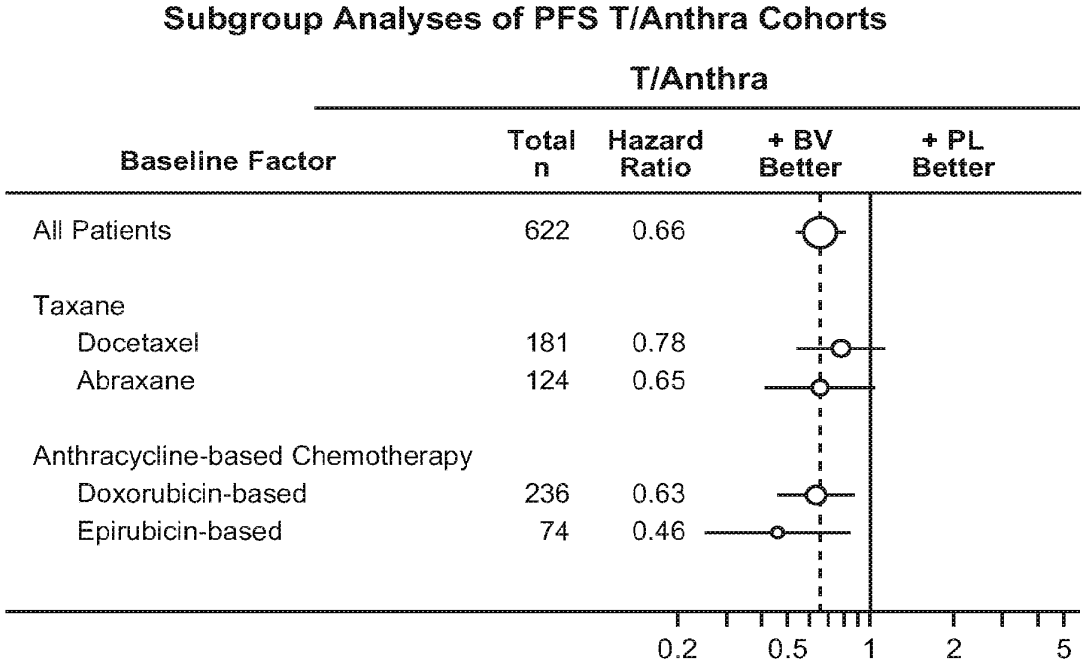


FIG. 6

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
4 October 2012 (04.10.2012)

WIPO | PCT

(10) International Publication Number
WO 2012/135405 A1

(51) International Patent Classification:
C07H 19/12 (2006.01)

(21) International Application Number:
PCT/US2012/031059

(22) International Filing Date:
29 March 2012 (29.03.2012)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/470,392 31 March 2011 (31.03.2011) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

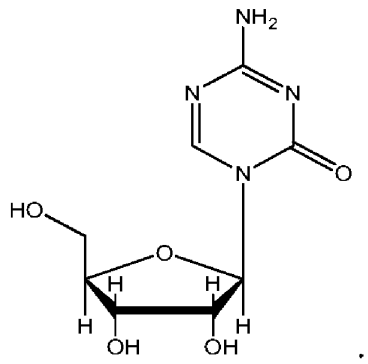
(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

(54) **Title**: SYSTHESIS OF 5-AZACYTIDINE



(57) **Abstract**: Provided herein are processes for the preparation of 5-azacytidine, useful for treating, preventing, and/or managing diseases or conditions including cancer, disorders related to abnormal cell proliferation, hematologic disorders, and myelodysplastic syndromes (MDS), wherein 5-azacytidine is represented by the structure:

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SYNTHESIS OF 5-AZACYTIDINE

[0001] This application claims priority to U.S. provisional patent application no. 61/470,392, filed March 31, 2011, the content of which is hereby incorporated by reference in its entirety.

I. FIELD

[0002] Provided herein are processes for the preparation of 5-azacytidine (also known as azacitidine). 5-Azacytidine is useful for treating, preventing, and/or managing diseases or conditions, including cancer, disorders related to abnormal cell proliferation, hematologic disorders, and myelodysplastic syndromes (MDS), among others.

II. BACKGROUND

[0003] Cancer is a major public health problem worldwide. In the United States alone, approximately 560,000 people died of cancer in 2006. *See, e.g.*, U.S. Mortality Data 2006, National Center for Health Statistics, Centers for Disease Control and Prevention (2009). Many types of cancer have been described in the medical literature. Examples include cancer of the blood, bone, skin, lung, colon, breast, prostate, ovary, brain, kidney, bladder, pancreas, and liver. The incidence of cancer continues to climb as the general population ages and as new forms of cancer develop. A continuing need exists for effective therapies to treat subjects with cancer.

[0004] Myelodysplastic syndromes (MDS) are a diverse group of hematopoietic cell disorders. MDS affect approximately 40,000–50,000 people in the U.S. and 75,000–85,000 people in Europe. MDS may be characterized by a cellular marrow with impaired morphology and maturation (dysmyelopoiesis), peripheral blood cytopenias, and a variable risk of progression to acute leukemia, resulting from ineffective blood cell production. *See, e.g.*, *The Merck Manual* 953 (17th ed. 1999); List *et al.*, *J. Clin. Oncol.* 8:1424 (1990).

[0005] MDS are grouped together because of the presence of dysplastic changes in one or more of the hematopoietic lineages including dysplastic changes in the myeloid, erythroid, and megakaryocytic series. These changes result in cytopenias in one or more of the three lineages. Patients afflicted with MDS may develop complications related to anemia, neutropenia (infections), and/or thrombocytopenia (bleeding). From about 10% to about 70% of patients with MDS may develop acute leukemia. In the early stages of

MDS, the main cause of cytopenias is increased programmed cell death (apoptosis). As the disease progresses and converts into leukemia, a proliferation of leukemic cells overwhelms the healthy marrow. The disease course differs, with some cases behaving as an indolent disease and others behaving aggressively with a very short clinical course that converts into an acute form of leukemia. The majority of people with higher risk MDS eventually experience bone marrow failure. Up to 50% of MDS patients succumb to complications, such as infection or bleeding, before progressing to AML.

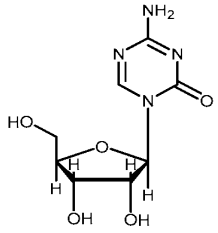
[0006] Primary and secondary MDS are defined by taking into account patients' prior history: previous treatments with chemotherapy, radiotherapy or professional exposure to toxic substances are factors delineating secondary MDS (sMDS) from primary MDS. Cytogenetically, one difference between the two groups is the complexity of abnormal karyotypes; single chromosome aberrations are typical for primary MDS, while multiple changes are more frequently seen in secondary disorders. Some drugs may have specific targets such as hydroxyurea for 17p and topoisomerases inhibitors for 11q23 and 21q22. The genetic changes in the malignant cells of MDS result mainly in the loss of genetic material, including probable tumor suppressor genes.

[0007] An international group of hematologists, the French–American–British (FAB) Cooperative Group, classified MDS into five subgroups, differentiating them from acute myeloid leukemia. *See, e.g.*, *The Merck Manual* 954 (17th ed. 1999); Bennett J. M., *et al.*, *Ann. Intern. Med.*, 103(4): 620–25 (1985); and Besa E. C., *Med. Clin. North Am.* 76(3): 599–617 (1992). An underlying trilineage dysplastic change in the bone marrow cells of the patients is found in all subtypes. Information is available regarding the pathobiology of MDS, certain MDS classification systems, and particular methods of treating and managing MDS. *See, e.g.*, U.S. Patent No. 7,189,740 (issued March 13, 2007), which is incorporated by reference herein in its entirety.

[0008] Nucleoside analogs have been used clinically for the treatment of viral infections and cancer. Most nucleoside analogs are classified as anti-metabolites. After they enter the cell, nucleoside analogs are successively phosphorylated to nucleoside 5'-mono-phosphates, di-phosphates, and tri-phosphates.

[0009] 5-Azacytidine (National Service Center designation NSC-102816; CAS Registry Number 320-67-2), also known as azacitidine, AZA, 4-amino-1-(3,4-dihydroxy-5-hydroxymethyl-tetrahydrofuran-2-yl)-1*H*-[1,3,5]triazin-2-one, 4-amino-1-β-D-ribofuranosyl-1,3,5-triazin-2(1*H*)-one, or 4-amino-1-β-D-ribofuranosyl-*S*-triazin-2(1*H*)-one, is currently marketed as the drug product VIDAZA®. 5-Azacytidine is a nucleoside

analog, more specifically a cytidine analog. 5-Azacytidine is an antagonist of its related natural nucleoside, cytidine. 5-Azacytidine is also an antagonist of deoxycytidine. A structural difference between 5-azacytidine and cytidine is the presence of a nitrogen at position 5 of the cytosine ring in place of a carbon. 5-Azacytidine may be defined as having the molecular formula $C_8H_{12}N_4O_5$, a molecular weight of about 244.2 grams per mole, and the following structure:



5-Azacytidine.

[0010] After its incorporation into replicating DNA, 5-azacytidine forms a covalent complex with DNA methyltransferases. DNA methyltransferases are responsible for *de novo* DNA methylation and for reproducing established methylation patterns in daughter DNA strands of replicating DNA. Inhibition of DNA methyltransferases by 5-azacytidine leads to DNA hypomethylation, thereby restoring normal functions to morphologically dysplastic, immature hematopoietic cells and cancer cells by re-expression of genes involved in normal cell cycle regulation, differentiation and death. The cytotoxic effects of 5-azacytidine cause the death of rapidly dividing cells, including cancer cells, that are no longer responsive to normal cell growth control mechanisms. 5-Azacytidine also incorporates into RNA. The cytotoxic effects of 5-azacytidine may result from multiple mechanisms, including inhibition of DNA, RNA and protein synthesis, incorporation into RNA and DNA, and activation of DNA damage pathways.

[0011] 5-Azacytidine has been tested in clinical trials and showed significant anti-tumor activity, such as, for example, in the treatment of myelodysplastic syndromes (MDS). *See, e.g.*, Aparicio *et al.*, *Curr. Opin. Invest. Drugs* 3(4): 627–33 (2002). 5-Azacytidine has undergone NCI-sponsored trials for the treatment of MDS and has been approved for treating all FAB subtypes of MDS. *See, e.g.*, Kornblith *et al.*, *J. Clin. Oncol.* 20(10): 2441–52 (2002); Silverman *et al., J. Clin. Oncol.* 20(10): 2429–40 (2002). 5-Azacytidine may alter the natural course of MDS by diminishing the transformation to AML through its cytotoxic activity and its inhibition of DNA methyltransferase. In a Phase III study, 5-azacytidine administered subcutaneously significantly prolonged

survival and time to AML transformation or death in subjects with higher-risk MDS. *See, e.g.,* P. Fenaux *et al.*, *Lancet Oncol.*, 2009, 10(3):223–32; Silverman *et al., Blood* 106(11): Abstract 2526 (2005).

[0012] 5-Azacytidine has been difficult to synthesize, particularly for manufacturing at large commercial scales, in part because of its instability in water. For example, the *s*-triazine ring of 5-azacytidine is prone to degradation in water. In aqueous solutions at neutral pH, hydration of the 5,6-imine double bond occurs rapidly, followed by bond cleavage to yield the formyl derivative, *N*-(formylamidino)-*N'*-β-D-ribofuranosylurea, which deformylates to give 1-β-D-ribofuranosyl-3-guanylurea irreversibly. *See, e.g.,* J. A. Beisler, *J. Med. Chem.*, 1978, 21(2):204-08; K. K. Chan *et al.*, *J. Pharm. Sci.* 1979, 68(7):807–12. In addition, the hydrolytic degradation of 5-azacytidine was studied as a function of pH, temperature, and buffer concentration. *See, e.g.,* R. E. Notari *et al.*, *Pharm. Sci.* 1975, 64(7):1148–57. At pH 1, the main degradation products were 5-azacytosine and 5-azauracil from the hydrolysis of 5-azacytidine. The instability of 5-azacytidine in water presents a challenge for the isolation and purification of 5-azacytidine from a solvent system that contains water, such as solvent systems used during the work-up stage of a reaction.

[0013] 5-Azacytidine was first prepared via a multi-step synthesis starting from peracetylated 1-glycosylisocyanate. *See* Piskala *et al.*, *Collect. Czech. Chem. Commun.*, 1964, 29:2060–76. This method involves a reactive starting material (isocyanate) with a controlled stereochemistry (1-β configuration), which is not suitable for the production of large scale batches of 5-azacytidine. Many other existing methods for preparing 5-azacytidine involve steps that are difficult to scale-up, or involve the use of expensive reagents. Other methods do not describe purification steps necessary to produce Active Pharmaceutical Ingredient (API) that meets the purity standards for human use, or give poor overall yields of the purified 5-azacytidine product.

[0014] U.S. Patent No. 7,038,038, issued May 2, 2006, which is incorporated herein by reference in its entirety, describes a process for preparing 5-azacytidine, comprising, *inter alia*, the steps of coupling a silylated 5-azacytosine with a protected β-D-ribofuranose in the presence of a non-metallic Lewis acid, such as trimethylsilyl trifluoromethanesulfonate (TMS-triflate), and deprotecting the product to give 5-azacytidine.

[0015] Metallic Lewis acids, such as stannic chloride, are generally less expensive and more readily available than non-metallic Lewis acids, such as TMS-triflate. However, the

use of metal-based reagents in the synthesis of API intended for human use generally requires appropriate purification steps to remove metal-based impurities in order to consistently control the levels of residual metals in the final API. For the production of a drug substance intended for use in humans, current Good Manufacturing Practices (cGMP) are applicable. Procedures need to be in place that can control the levels of impurities and ensure that API batches are produced which consistently meet their predetermined specifications. For example, in a GMP environment, it is not acceptable to have one batch having a heavy metal content that is within a desired specification, and then have a batch run under similar circumstances having heavy metal impurities well over the desired specification.

[0016] A great need remains for a process to prepare pure 5-azacytidine suitable for human use, particularly on a commercial scale, that is, *inter alia*, safe, scalable, efficient, economically viable, and/or having other potential advantages.

[0017] Citation of any references in this Section of the application is not to be construed as an admission that such reference is prior art to the present application.

III. SUMMARY

[0018] Provided herein are, *inter alia*, safe, efficient, cost effective, and/or readily scaleable processes useful for the production of 5-azacytidine, or a salt, solvate, hydrate, or polymorph thereof. In one embodiment, provided herein are processes useful for the production of 5-azacytidine, or a salt, solvate, hydrate, or polymorph thereof, that is substantially pure. In one embodiment, provided herein are processes useful for the production of 5-azacytidine, or a salt, solvate, hydrate, or polymorph thereof, that is substantially chemically pure. In one embodiment, provided herein are processes useful for the production of 5-azacytidine, or a salt, solvate, hydrate, or polymorph thereof, that is substantially physically pure. In one embodiment, provided herein are processes useful for the production of 5-azacytidine, or a salt, solvate, hydrate, or polymorph thereof, suitable for use in humans.

[0019] In one embodiment, provided herein are processes for preparing 5-azacytidine, or a salt, solvate, hydrate, or polymorph thereof, comprising the steps of reacting a silylated 5-azacytosine with a protected β-D-ribofuranose in the presence of a metallic Lewis acid to yield a protected 5-azacytidine, deprotecting the protected 5-azacytidine to yield 5-azacytidine, and purifying the 5-azacytidine to yield 5-azacytidine, or a salt, solvate, hydrate, or polymorph thereof, that is substantially free of one or more impurities,

including but not limited to, a metal-based impurity.

[0020] In one embodiment, provided herein is a salt of 5-azacytidine, including, but not limited to, hydrochloric acid salt, sulfuric acid salt, hydrobromic acid salt, and methanesulfonic acid salt. In one embodiment, provided herein is 5-azacytidine hydrochloric acid salt. In one embodiment, provided herein is a salt of 5-azacytidine that is substantially free of one or more impurities, such as for example, a metal-based impurity. In one embodiment, provided herein is a pharmaceutically acceptable salt of 5-azacytidine, including, but not limited to hydrochloric acid salt, sulfuric acid salt, hydrobromic acid salt, and methanesulfonic acid salt. In one embodiment, provided herein is a pharmaceutically acceptable salt of 5-azacytidine that is substantially free of one or more impurities, such as for example, a metal-based impurity.

[0021] In specific embodiments, provided herein are processes for preparing 5-azacytidine, or a salt, solvate, hydrate, or polymorph thereof, comprising any one, two, three, four, five, or six of the steps of:

(a) reacting 5-azacytosine with a silylating reagent to yield a silylated 5-azacytosine;

(b) reacting the silylated 5-azacytosine with an acyl protected β-D-ribofuranose in the presence of a metallic Lewis acid; and quenching the reaction with water and at least one neutralizing reagent to yield a protected 5-azacytidine;

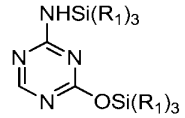
(c) reacting the protected 5-azacytidine with a base, selected from the group consisting of alkoxide, ammonia, and tetra-substituted ammonium hydroxide, in an alcohol to yield 5-azacytidine;

(d) contacting the 5-azacytidine from step (c) with an acid in an organic solvent to yield a salt of 5-azacytidine;

(e) contacting the salt of 5-azacytidine from step (d) with a base in an organic solvent to yield 5-azacytidine as a free base; and

(f) re-crystallizing the 5-azacytidine from step (e).

[0022] In one embodiment, the silylated 5-azacytosine is a compound of formula (A):



(A),

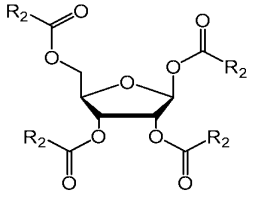
wherein each R_1 is independently optionally substituted C_1–C_{10} alkyl, optionally substituted C_3–C_{10} cycloalkyl, or optionally substituted C_6–C_{10} aryl. In some embodiments, R_1 is straight chain alkyl, branched alkyl, cycloalkyl, or aryl, including but

not limited to, methyl, ethyl, *i*-propyl, *t*-butyl, phenyl, xylyl, and benzyl. In some embodiments, R_1 is methyl.

[0023] In one embodiment, the silylating reagent used in step (a) is a trimethylsilyl (TMS) reagent, including but not limited to, hexamethyldisilazane (HMDS) and chlorotrimethylsilane (TMSCl).

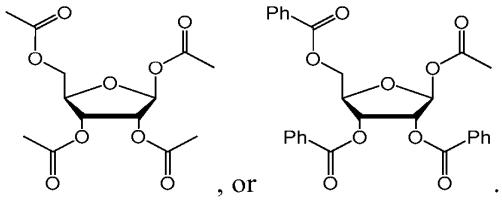
[0024] In one embodiment, the silylation reaction of step (a) is carried out in the presence of ammonium sulfate. In one embodiment, the silylation reaction of step (a) is carried out at elevated temperature. In one embodiment, the silylation reaction of step (a) is carried out under an inert atmosphere. In some embodiments, the silylated 5-azacytosine is isolated as a solid. In other embodiments, the silylated 5-azacytosine is used directly in step (b) without isolation.

[0025] In one embodiment, the acyl protected β-D-ribofuranose is a compound of formula (B):



(B),

wherein each R_2 is independently hydrogen, optionally substituted C_1–C_{10} alkyl, optionally substituted C_3–C_{10} cycloalkyl, or optionally substituted C_6–C_{10} aryl. In some embodiments, R_2 is optionally substituted methyl or optionally substituted phenyl. In some embodiments, R_2 is methyl. In some embodiments, R_2 is phenyl. In some embodiments, the acyl protected β-D-ribofuranose is:



, or

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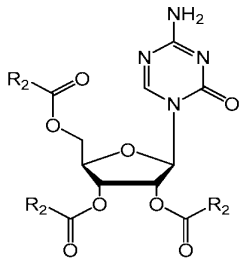
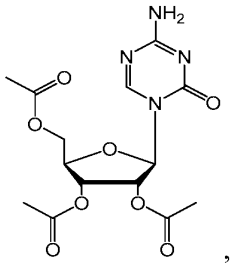
[0026] In one embodiment, the metallic Lewis acid is stannic chloride or ferric chloride. In one embodiment, the metallic Lewis acid is stannic chloride. In one embodiment, the metallic Lewis acid is ferric chloride.

[0027] In one embodiment, the protected 5-azacytidine is a compound of formula (C):



(C),

wherein each R_2 is independently hydrogen, optionally substituted C_1–C_{10} alkyl, optionally substituted C_3–C_{10} cycloalkyl, or optionally substituted C_6–C_{10} aryl. In some embodiments, R_2 is optionally substituted methyl or optionally substituted phenyl. In some embodiments, R_2 is methyl. In some embodiments, R_2 is phenyl. In some embodiments, the protected 5-azacytidine is: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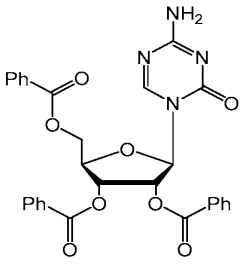
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[0028] In one embodiment, the reaction of step (b) is carried out in a solvent with low water solubility. In some embodiments, the reaction of step (b) is carried out in dichloromethane. In one embodiment, the reaction of step (b) is carried out at a temperature of less than about 10˚C. In one embodiment, the reaction of step (b) is carried out at a temperature of less than about 5˚C. In one embodiment, the reaction of step (b) is carried out at a temperature of greater than about –20˚C. In one embodiment, the reaction of step (b) is carried out at a temperature of greater than about –10˚C. In one embodiment, the reaction of step (b) is carried out at a temperature of greater than about 0˚C. In one embodiment, the reaction of step (b) is carried out at a temperature of between about 0˚C and about 5˚C.

[0029] In one embodiment, the neutralizing reagent in step (b) is an inorganic reagent. In one embodiment, the neutralizing reagent in step (b) is an inorganic base. In one embodiment, the neutralizing reagent in step (b) is an inorganic salt. In one embodiment, the neutralizing reagent in step (b) is a carbonate or bicarbonate salt, or a mixture thereof. In one embodiment, the neutralizing reagent in step (b) is sodium bicarbonate. In one

embodiment, the neutralizing reagent in step (b) is sodium carbonate.

[0030] In one embodiment, the reaction of step (b) is quenched with water and one or more neutralizing reagents(s) to yield a quenched composition. In one embodiment, the reaction of step (b) is quenched at a temperature of less than about 10°C. In one embodiment, the quenched composition of the reaction of step (b) is filtered. In one embodiment, the quenching composition of the reaction of step (b) is filtered at a temperature of less than about 10°C. In one embodiment, the filtrate of the quenched composition of the reaction of step (b) is washed with an aqueous EDTA (ethylenediaminetetraacetic acid) salt solution. In one embodiment, the filtrate of the quenched composition of the reaction of step (b) is washed with an aqueous EDTA disodium salt solution.

[0031] In one embodiment, the base in step (c) is an alkoxide. In one embodiment, the base in step (c) is a sodium alkoxide. In one embodiment, the base in step (c) is sodium methoxide. In one embodiment, the alcohol in step (c) is methanol. In one embodiment, the 5-azacytidine from step (c) is collected by filtration. In one embodiment, the 5-azacytidine from step (c) is washed with a non-aqueous solvent, including but not limited to, an alcohol, such as methanol.

[0032] In one embodiment, the acid in step (d) is an organic or an inorganic acid, including, but not limited to, hydrochloric acid, hydrobromic acid, sulfuric acid, and methanesulfonic acid. In some embodiments, the acid used in step (d) is hydrochloric acid. In one embodiment, the organic solvent in step (d) is an alcohol. In some embodiments, the organic solvent in step (d) is methanol. In some embodiments, the organic solvent in step (d) is isopropanol.

[0033] In one embodiment, the salt of 5-azacytidine from step (d) is collected by filtration. In one embodiment, the salt of 5-azacytidine from step (d) is washed with an organic solvent, including but not limited to, an alcohol, such as methanol.

[0034] In one embodiment, the base in step (e) is an organic base, including but not limited to, triethylamine, diisopropylethyl amine, pyridine, diisopropylamine, 2,6-lutidine, *N*-methylmorpholine, and *N,N*-dicyclohexylmethyl amine. In some embodiments, the base in step (e) is triethylamine. In one embodiment, the organic solvent in step (e) is an alcohol. In some embodiments, the organic solvent in step (e) is methanol. In one embodiment, the free base of 5-azacytidine from step (e) is collected by filtration. In one embodiment, the free base of 5-azacytidine from step (e) is washed with an organic solvent, including but not limited to, an alcohol, such as methanol. In one embodiment,

the free base of 5-azacytidine from step (e) is substantially free of one or more impurities, including but not limited to, a metal-based impurity and an acidic salt counter ion, such as for example, chloride.

[0035] In one embodiment, step (f) comprises the steps of:

(1) dissolving 5-azacytidine free base from step (e) in dimethylsulfoxide (DMSO) at a temperature sufficient to allow the 5-azacytidine to dissolve; and optionally filtering the solution to remove insoluble particles;

(2) adding an anti-solvent to the solution of step (1); and

(3) cooling the mixture of step (2) wherein 5-azacytidine re-crystallizes.

[0036] In one embodiment, the optional filtration in step (f)(1) is carried out at an elevated temperature, such as for example, a temperature greater than about 85°C. In one embodiment, the anti-solvent of step (f)(2) is an alcohol. In one embodiment, the anti-solvent of step (f)(2) is methanol.

[0037] In one embodiment, step (f) further comprises the steps of:

(4) collecting the re-crystallized 5-azacytidine from step (3) by filtration; and

(5) drying the 5-azacytidine from step (4) under vacuum.

[0038] In one embodiment, provided herein are processes for preparing 5-azacytidine, or a pharmaceutically acceptable salt, solvate, hydrate, or polymorph thereof, for treating, preventing, and/or managing diseases or conditions including cancer, disorders related to abnormal cell proliferation, hematologic disorders, and myelodysplastic syndromes (MDS), among others.

IV. BRIEF DESCRIPTION OF THE FIGURES

[0039] **FIG. 1** represents an Infrared (IR) spectrum of 5-azacytidine.

[0040] **FIG. 2** represents a Differential Scanning Calorimetry (DSC) plot of 5-azacytidine.

[0041] **FIG. 3** represents an X-Ray Powder Diffraction (XRPD) pattern of 5-azacytidine.

[0042] **FIG. 4** represents an IR spectrum of 5-azacytidine.

[0043] **FIG. 5** represents a DSC plot of 5-azacytidine.

[0044] **FIG. 6** represents an XRPD pattern of 5-azacytidine.

[0045] **FIG. 7** represents an IR spectrum of a 5-azacytidine mono-hydrochloride salt.

[0046] **FIG. 8** represents a DSC plot of the a 5-azacytidine mono-hydrochloride salt.

[0047] **FIG. 9** represents an XRPD pattern of a 5-azacytidine mono-hydrochloride salt.

[0048] **FIG. 10** represents an XRPD pattern of a 5-azacytidine mono-hydrochloride salt.

[0049] **FIG. 11** represents an IR spectrum of a methanol solvate of 5-azacytidine hemisulfate salt.

[0050] **FIG. 12** represents a DSC plot of a methanol solvate of 5-azacytidine hemisulfate salt.

[0051] **FIG. 13** represents an XRPD pattern of a methanol solvate of 5-azacytidine hemisulfate salt.

[0052] **FIG. 14** represents an IR spectrum of a methanol solvate of 5-azacytidine mesylate salt.

[0053] **FIG. 15** represents a DSC plot of a methanol solvate of 5-azacytidine mesylate salt.

[0054] **FIG. 16** represents an XRPD pattern of a methanol solvate of 5-azacytidine mesylate salt.

[0055] **FIG. 17** represents an IR spectrum of a 5-azacytidine hydrobromide salt.

[0056] **FIG. 18** represents a DSC plot of a 5-azacytidine hydrobromide salt.

[0057] **FIG. 19** represents an XRPD pattern of a 5-azacytidine hydrobromide salt.

[0058] **FIG. 20** represents an IR spectrum of 2',3',5'-triacetyl-5-azacytidine.

V. DETAILED DESCRIPTION

[0059] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as those commonly understood by one of ordinary skill in the art. All publications and patents referred to herein are incorporated by reference herein in their entireties.

A. Definitions

[0060] As used herein, and unless otherwise specified, the term "alkyl" refers to a linear or branched saturated monovalent hydrocarbon radical, wherein the alkyl may optionally be substituted with one or more substituents. The term "alkyl" also encompasses both linear and branched alkyl, unless otherwise specified. In certain embodiments, the alkyl is a linear saturated monovalent hydrocarbon radical that has 1 to 20 (C_{1-20}), 1 to 15 (C_{1-15}), 1 to 12 (C_{1-12}), 1 to 10 (C_{1-10}), or 1 to 6 (C_{1-6}) carbon atoms, or

branched saturated monovalent hydrocarbon radical of 3 to 20 (C_{3-20}), 3 to 15 (C_{3-15}), 3 to 12 (C_{3-12}), 3 to 10 (C_{3-10}), or 3 to 6 (C_{3-6}) carbon atoms. As used herein, linear C_{1-6} and branched C_{3-6} alkyl groups are also referred as "lower alkyl." Examples of alkyl groups include, but are not limited to, methyl, ethyl, propyl (including all isomeric forms), *n*-propyl, *i*-propyl, butyl (including all isomeric forms), *n*-butyl, *i*-butyl, *t*-butyl, pentyl (including all isomeric forms), and hexyl (including all isomeric forms). For example, C_{1-6} alkyl refers to a linear saturated monovalent hydrocarbon radical of 1 to 6 carbon atoms or a branched saturated monovalent hydrocarbon radical of 3 to 6 carbon atoms.

[0061] As used herein, and unless otherwise specified, the term "cycloalkyl" refers to a cyclic saturated bridged and/or non-bridged monovalent hydrocarbon radical, which may be optionally substituted one or more substituents as described herein. In certain embodiments, the cycloalkyl has from 3 to 20 (C_{3-20}), from 3 to 15 (C_{3-15}), from 3 to 12 (C_{3-12}), from 3 to 10 (C_{3-10}), or from 3 to 7 (C_{3-7}) carbon atoms. Examples of cycloalkyl groups include, but are not limited to, cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, decalinyl, and adamantyl.

[0062] As used herein, and unless otherwise specified, the term "aryl" refers to a monocyclic aromatic group and/or multicyclic monovalent aromatic group that contain at least one aromatic hydrocarbon ring. In certain embodiments, the aryl has from 6 to 20 (C_{6-20}), from 6 to 15 (C_{6-15}), or from 6 to 10 (C_{6-10}) ring atoms. Examples of aryl groups include, but are not limited to, phenyl, naphthyl, fluorenyl, azulenyl, anthryl, phenanthryl, pyrenyl, biphenyl, and terphenyl. Aryl also refers to bicyclic or tricyclic carbon rings, where one of the rings is aromatic and the others of which may be saturated, partially unsaturated, or aromatic, for example, dihydronaphthyl, indenyl, indanyl, or tetrahydronaphthyl (tetralinyl). In certain embodiments, aryl may also be optionally substituted with one or more substituents.

[0063] As used herein, and unless otherwise specified, the term "halo," "halogen," or "halide" refers to fluorine, chlorine, bromine, and/or iodine.

[0064] As used herein, and unless otherwise specified, the term "hydrogen" encompasses proton (^1H), deuterium (^2H), tritium (^3H), and/or mixtures thereof.

[0065] As used herein, and unless otherwise specified, the term "optionally substituted" is intended to mean that a group, such as an alkyl, alkenyl, alkynyl, cycloalkyl, aryl, aralkyl, heteroaryl, or heterocyclyl, may be substituted with one or more substituents independently selected from, *e.g.*, (a) C_{1-6} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, C_{3-7} cycloalkyl, C_{6-14} aryl, C_{7-15} aralkyl, heteroaryl, and heterocyclyl, each optionally

substituted with one or more (in specific embodiments, one, two, three, or four) substituents Q^1; and (b) halo, cyano (–CN), nitro ($–NO_2$), $–C(O)R^a$, $–C(O)OR^a$, $–C(O)NR^bR^c$, $–C(NR^a)NR^bR^c$, $–OR^a$, $–OC(O)R^a$, $–OC(O)OR^a$, $–OC(O)NR^bR^c$, $–OC(=NR^a)NR^bR^c$, $–OS(O)R^a$, $–OS(O)_2R^a$, $–OS(O)NR^bR^c$, $–OS(O)_2NR^bR^c$, $–NR^bR^c$, $–NR^aC(O)R^d$, $–NR^aC(O)OR^d$, $–NR^aC(O)NR^bR^c$, $–NR^aC(=NR^d)NR^bR^c$, $–NR^aS(O)R^d$, $–NR^aS(O)_2R^d$, $–NR^aS(O)NR^bR^c$, $–NR^aS(O)_2NR^bR^c$, $–SR^a$, $–S(O)R^a$, $–S(O)_2R^a$, $–S(O)NR^bR^c$, and $–S(O)_2NR^bR^c$, wherein each R^a, R^b, R^c, and R^d is independently (i) hydrogen; (ii) C_{1-6} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, C_{3-7} cycloalkyl, C_{6-14} aryl, C_{7-15} aralkyl, heteroaryl, or heterocyclyl, each optionally substituted with one or more (in specific embodiments, one, two, three, or four) substituents Q^1; or (iii) R^b and R^c together with the N atom to which they are attached form heteroaryl or heterocyclyl, optionally substituted with one or more (in specific embodiments, one, two, three, or four) substituents Q^1. As used herein, all groups that can be substituted are "optionally substituted," unless otherwise specified.

[0066] In specific embodiments, each Q^1 is independently selected from the group consisting of (a) cyano, halo, and nitro; and (b) C_{1-6} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, C_{3-7} cycloalkyl, C_{6-14} aryl, C_{7-15} aralkyl, heteroaryl, and heterocyclyl; and (c) $–C(O)R^e$, $–C(O)OR^e$, $–C(O)NR^fR^g$, $–C(NR^e)NR^fR^g$, $–OR^e$, $–OC(O)R^e$, $–OC(O)OR^e$, $–OC(O)NR^fR^g$, $–OC(=NR^e)NR^fR^g$, $–OS(O)R^e$, $–OS(O)_2R^e$, $–OS(O)NR^fR^g$, $–OS(O)_2NR^fR^g$, $–NR^fR^g$, $–NR^eC(O)R^h$, $–NR^eC(O)OR^h$, $–NR^eC(O)NR^fR^g$, $–NR^eC(=NR^h)NR^fR^g$, $–NR^eS(O)R^h$, $–NR^eS(O)_2R^h$, $–NR^eS(O)NR^fR^g$, $–NR^eS(O)_2NR^fR^g$, $–SR^e$, $–S(O)R^e$, $–S(O)_2R^e$, $–S(O)NR^fR^g$, and $–S(O)_2NR^fR^g$; wherein each R^e, R^f, R^g, and R^h is independently (i) hydrogen; (ii) C_{1-6} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, C_{3-7} cycloalkyl, C_{6-14} aryl, C_{7-15} aralkyl, heteroaryl, or heterocyclyl; or (iii) R^f and R^g together with the N atom to which they are attached form heteroaryl or heterocyclyl.

[0067] As used herein, and unless otherwise specified, the term "alkenyl" refers to a linear or branched monovalent hydrocarbon radical, which contains one or more (in specific embodiments, one to five) carbon-carbon double bonds. The alkenyl may be optionally substituted with one or more substituents. The term "alkenyl" also encompasses radicals having "*cis*" and "*trans*" configurations, or alternatively, "*E*" and "*Z*" configurations, as appreciated by those of ordinary skill in the art. As used herein, the term "alkenyl" encompasses both linear and branched alkenyl, unless otherwise specified. For example, C_{2-6} alkenyl refers to a linear unsaturated monovalent hydrocarbon radical of 2 to 6 carbon atoms or a branched unsaturated monovalent hydrocarbon radical of 3 to 6

carbon atoms. In certain embodiments, the alkenyl is a linear monovalent hydrocarbon radical of 2 to 20 (C_{2-20}), 2 to 15 (C_{2-15}), 2 to 12 (C_{2-12}), 2 to 10 (C_{2-10}), or 2 to 6 (C_{2-6}) carbon atoms, or a branched monovalent hydrocarbon radical of 3 to 20 (C_{3-20}), 3 to 15 (C_{3-15}), 3 to 12 (C_{3-12}), 3 to 10 (C_{3-10}), or 3 to 6 (C_{3-6}) carbon atoms. Examples of alkenyl groups include, but are not limited to, ethenyl, propen-1-yl, propen-2-yl, allyl, butenyl, and 4-methylbutenyl.

[0068] As used herein, and unless otherwise specified, the term "alkynyl" refers to a linear or branched monovalent hydrocarbon radical, which contains one or more (in specific embodiments, one to five) carbon-carbon triple bonds. The alkynyl may be optionally substituted one or more substituents. The term "alkynyl" also encompasses both linear and branched alkynyl, unless otherwise specified. In certain embodiments, the alkynyl is a linear monovalent hydrocarbon radical of 2 to 20 (C_{2-20}), 2 to 15 (C_{2-15}), 2 to 12 (C_{2-12}), 2 to 10 (C_{2-10}), or 2 to 6 (C_{2-6}) carbon atoms, or a branched monovalent hydrocarbon radical of 3 to 20 (C_{3-20}), 3 to 15 (C_{3-15}), 3 to 12 (C_{3-12}), 3 to 10 (C_{3-10}), or 3 to 6 (C_{3-6}) carbon atoms. Examples of alkynyl groups include, but are not limited to, ethynyl (–C≡CH) and propargyl (–CH_2C≡CH). For example, C_{2-6} alkynyl refers to a linear unsaturated monovalent hydrocarbon radical of 2 to 6 carbon atoms or a branched unsaturated monovalent hydrocarbon radical of 3 to 6 carbon atoms.

[0069] As used herein, and unless otherwise specified, the term "arylalkyl" or "aralkyl" refers to a monovalent alkyl group substituted with aryl. In certain embodiments, both alkyl and aryl may be optionally substituted with one or more substituents.

[0070] As used herein, and unless otherwise specified, the term "heteroaryl" refers to a monocyclic aromatic group and/or multicyclic aromatic group that contain at least one aromatic ring, wherein at least one aromatic ring contains one or more heteroatoms independently selected from O, S, and N. Each ring of a heteroaryl group can contain one or two O atoms, one or two S atoms, and/or one to four N atoms, provided that the total number of heteroatoms in each ring is four or less and each ring contains at least one carbon atom. In certain embodiments, the heteroaryl has from 5 to 20, from 5 to 15, or from 5 to 10 ring atoms. Examples of monocyclic heteroaryl groups include, but are not limited to, furanyl, imidazolyl, isothiazolyl, isoxazolyl, oxadiazolyl, oxazolyl, pyrazinyl, pyrazolyl, pyridazinyl, pyridyl, pyrimidinyl, pyrrolyl, thiadiazolyl, thiazolyl, thienyl, tetrazolyl, triazinyl, and triazolyl. Examples of bicyclic heteroaryl groups include, but are not limited to, benzofuranyl, benzimidazolyl, benzoisoxazolyl, benzopyranyl,

benzothiadiazolyl, benzothiazolyl, benzothiophenyl, benzotriazolyl, benzoxazolyl, furopyridyl, imidazopyridinyl, imidazothiazolyl, indolizinyl, indolyl, indazolyl, isobenzofuranyl, isobenzothienyl, isoindolyl, isoquinolinyl, isothiazolyl, naphthyridinyl, oxazolopyridinyl, phthalazinyl, pteridinyl, purinyl, pyridopyridyl, pyrrolopyridyl, quinolinyl, quinoxalinyl, quinazolinyl, thiadiazolopyrimidyl, and thienopyridyl. Examples of tricyclic heteroaryl groups include, but are not limited to, acridinyl, benzindolyl, carbazolyl, dibenzofuranyl, perimidinyl, phenanthrolinyl, phenanthridinyl, phenarsazinyl, phenazinyl, phenothiazinyl, phenoxazinyl, and xanthenyl. In certain embodiments, heteroaryl groups may be optionally substituted with one or more substituents.

[0071] As used herein, and unless otherwise specified, the term "heterocycloalkyl," "heterocyclyl," or "heterocyclic" refers to a monocyclic non-aromatic ring system and/or multicyclic ring system that contains at least one non-aromatic ring, wherein one or more of the non-aromatic ring atoms are heteroatoms independently selected from O, S, or N; and the remaining ring atoms are carbon atoms. In certain embodiments, the heterocyclyl or heterocyclic group has from 3 to 20, from 3 to 15, from 3 to 10, from 3 to 8, from 4 to 7, or from 5 to 6 ring atoms. In certain embodiments, the heterocyclyl is a monocyclic, bicyclic, tricyclic, or tetracyclic ring system, which may include a fused or bridged ring system, and in which the nitrogen or sulfur atoms may be optionally oxidized, the nitrogen atoms may be optionally quaternized, and some rings may be partially or fully saturated, or aromatic. The heterocyclyl may be attached to the main structure at any heteroatom or carbon atom which results in the creation of a stable compound. Examples of such heterocyclic radicals include, but are not limited to, azepinyl, benzodioxanyl, benzodioxolyl, benzofuranonyl, benzopyranonyl, benzopyranyl, benzotetrahydrofuranyl, benzotetrahydrothienyl, benzothiopyranyl, benzoxazinyl, β-carbolinyl, chromanyl, chromonyl, cinnolinyl, coumarinyl, decahydroisoquinolinyl, dihydrobenzisothiazinyl, dihydrobenzisoxazinyl, dihydrofuryl, dihydroisoindolyl, dihydropyranyl, dihydropyrazolyl, dihydropyrazinyl, dihydropyridinyl, dihydropyrimidinyl, dihydropyrrolyl, dioxolanyl, 1,4-dithianyl, furanonyl, imidazolidinyl, imidazolinyl, indolinyl, isobenzotetrahydrofuranyl, isobenzotetrahydrothienyl, isochromanyl, isocoumarinyl, isoindolinyl, isothiazolidinyl, isoxazolidinyl, morpholinyl, octahydroindolyl, octahydroisoindolyl, oxazolidinonyl, oxazolidinyl, oxiranyl, piperazinyl, piperidinyl, 4-piperidonyl, pyrazolidinyl, pyrazolinyl, pyrrolidinyl, pyrrolinyl, quinuclidinyl, tetrahydrofuryl, tetrahydroisoquinolinyl, tetrahydropyranyl, tetrahydrothienyl, thiamorpholinyl, thiazolidinyl, tetrahydroquinolinyl, and 1,3,5-

trithianyl. In certain embodiments, heterocyclic may be optionally substituted with one or more substituents.

[0072] As used herein, and unless otherwise specified, the term "methylene" refers to a divalent $–CH_2–$ group.

[0073] As used herein, and unless otherwise specified, the term "carbonyl" refers to a divalent –C(=O)– group.

[0074] As used herein, and unless otherwise specified, the term "heteroalkyl" or "heteroalkyl group" refers to a univalent group derived from an alkyl group, where at least one methylene group is replaced by a heteroatom or a hetero-group such as O, S, or NR, where R is H or an organic group.

[0075] As used herein, and unless otherwise specified, the term "organic group" refers to a group containing at least one carbon atom. Examples of the organic group include, but are not limited to, alkyl, alkenyl, alkynyl, carboxyl, acyl, cycloalkyl, aryl, heteroaryl, heteroalkyl, and heterocycloalkyl.

[0076] As used herein, and unless otherwise specified, the term "alkoxy" or "alkoxy group" refers to an alkyl group that is linked to another group via an oxygen atom (*i.e.*, –O–alkyl). An alkoxy group can be unsubstituted or substituted with one or more suitable substituents. Examples of alkoxy groups include, but are not limited to, $(C_1–C_6)$alkoxy groups, such as –O–methyl, –O–ethyl, –O–propyl, –O–isopropyl, –O–2–methyl–1–propyl, –O–2–methyl–2–propyl, –O–2–methyl–1–butyl, –O–3–methyl–1–butyl, –O–2–methyl–3–butyl, –O–2,2–dimethyl–1–propyl, –O–2–methyl–1–pentyl, –O–3–methyl–1–pentyl, –O–4–methyl–1–pentyl, –O–2–methyl–2–pentyl, –O–3–methyl–2–pentyl, –O–4–methyl–2–pentyl, –O–2,2–dimethyl–1–butyl, –O–3,3–dimethyl–1–butyl, –O–2–ethyl–1–butyl, –O–butyl, –O–isobutyl, –O–*t*–butyl, –O–pentyl, –O–isopentyl, –O–neopentyl, and –O–hexyl. An alkoxy group can be unsubstituted or substituted with one or two suitable substituents. In some embodiments, the alkyl chain of an alkyloxy group is straight or branched, and has from 1 to 8 carbon atoms, referred to herein as "$(C_1$-$C_8)$alkoxy".

[0077] As used herein, and unless otherwise specified, the term "aryloxy" or "aryloxy group" refers to an O-aryl group, wherein aryl is as defined herein elsewhere. An aryloxy group can be unsubstituted or substituted with one or two suitable substituents. In some embodiments, the aryl ring of an aryloxy group is a monocyclic ring, wherein the ring comprises 6 carbon atoms, referred to herein as "(C_6)aryloxy."

[0078] As used herein, and unless otherwise specified, the term "alkoxycarbonyl" or "alkoxycarbonyl group" refers to a monovalent group of the formula –C(=O)–alkoxy. In

some embodiments, the hydrocarbon chain of an alkoxycarbonyl group is straight or branched, and has from 1 to 8 carbon atoms, referred to herein as a "lower alkoxycarbonyl" group.

[0079] As used herein, and unless otherwise specified, the term "alkylsulfanyl" or "alkylsulfanyl group" refers to a monovalent group of the formula –S–alkyl. In some embodiments, the hydrocarbon chain of an alkylsulfanyl group is straight or branched, and has from 1 to 8 carbon atoms, referred to herein as a "lower alkylsulfanyl" group.

[0080] As used herein, and unless otherwise specified, the term "acyloxy" or "acyloxy group" refers to a monovalent group of the formula –O–C(=O)–alkyl or –O–C(=O)–aryl, wherein alkyl and aryl are as defined herein elsewhere.

[0081] As used herein, and unless otherwise specified, the term "acyl" or "acyl group" refers to a monovalent group of the formula –C(=O)H, –C(=O)–alkyl, or –C(=O)–aryl, wherein alkyl and aryl are as defined herein elsewhere.

[0082] When a compound provided herein contains one or more acidic or basic moieties, the compound may exist as a salt. As used herein, and unless otherwise specified, the term "salt" or "salts" of a compound refers to salt(s) of a compound having basic or acidic groups, and the salts are prepared from the compound and one or more acids, including inorganic acids and organic acids; or one or more bases, including inorganic bases and organic bases. In certain embodiments, the compounds provided herein are basic in nature and are capable of forming a wide variety of salts with various inorganic or organic acids. The acids that may be used to prepare salts of such basic compounds are described herein elsewhere. In certain embodiments, the compounds provided herein are acidic in nature and are capable of forming a wide variety of salts with various inorganic or organic bases. Non-limiting examples of such salts with inorganic bases include alkali metal or alkaline earth metal salts. In certain embodiments, the salt of a compound provided herein comprises one or more acidic or basic counter-ions, including, but not limited to: acetate, ascorbate, benzenesulfonate, benzoate, bicarbonate, bitartrate, bromide, calcium edetate, camsylate, carbonate, chloride, iodide, citrate, dihydrochloride, edetate, edisylate, estolate, esylate, fumarate, gluceptate, gluconate, glutamate, glycollylarsanilate, hexylresorcinate, hydrabamine, hydroxynaphthoate, isethionate, lactate, lactobionate, malate, maleate, malonate, mandelate, mesylate, methanesulfonate, muscate, napsylate, nitrate, oxalate, panthothenate, phosphate, diphosphate, polygalacturonate, salicylate, stearate, succinate, sulfate, bisulfate, hemisulfate, sulfite, tannate, tartrate, teoclate, triethiodide, and/or pamoate, and the like; or

lithium, sodium, potassium, magnesium, calcium, zinc, iron, and/or ammonium ions, and the like; or *N,N*-dicyclohexylmethyl amine, diisopropylamine, diisopropylethyl amine, ethanolamine, 2,6-lutidine, *N*-methylmorpholine, pyridine, and/or triethylamine, and the like; or amino acids, and/or protected amino acids, and the like.

[0083] When a compound provided herein contains an acidic or basic moiety, it may also be provided as a pharmaceutically acceptable salt (*See*, *e.g.,* "Handbook of Pharmaceutical Salts, Properties, and Use," Stahl and Wermuth, Ed.; Wiley-VCH and VHCA, Zurich, 2002). As used herein, and unless otherwise specified, the term "pharmaceutically acceptable salts" refers to salts prepared from pharmaceutically acceptable non-toxic acids, including inorganic acids and organic acids, or pharmaceutically acceptable non-toxic bases, including inorganic bases and organic bases.

[0084] Suitable acids for use in the preparation of "salts" or "pharmaceutically acceptable salts" include, but are not limited to, acetic, 2,2-dichloroacetic, acylated amino, adipic, alginic, anthranilic, ascorbic, aspartic, L-aspartic, D-aspartic, benzenesulfonic, benzoic, 4-acetamidobenzoic, boric, camphoric, (+)-camphoric, (-)-camphoric, camphorsulfonic, (1*R*)-(-)-10-camphorsulfonic, (1*S*)-(+)-10-camphorsulfonic, capric, caproic, caprylic, cinnamic, citric, cyclamic, cyclohexanesulfamic, dodecylsulfuric, ethane-1,2-disulfonic, ethenesulfonic, 2-hydroxy-ethanesulfonic, formic, fumaric, furoic, galactaric, galacturonic, gentistic, glucarenic, glucoheptonic, gluconic, D-gluconic, L-gluconic, glucuronic, D-glucuronic, L-glucuronic, glutamic, D-glutamic, L-glutamic, glutaric, oxoglutaric, α-oxoglutaric, β-oxoglutaric, glycolic, glycidic, hippuric, hydrobromic, hydrochloric, hydroiodic, isethionic, lactic, D-lactic, L-lactic, lactobionic, lauric, maleic, malic, D-malic, L-malic, malonic, mandelic, (+)-mandelic, (-)-mandelic, methanesulfonic, mucic, naphthalene-2-sulfonic, naphthalene-1,5-disulfonic, 1-hydroxy-2-naphthoic, nicotinic, nitric, oleic, orotic, oxalic, palmitic, pamoic, pantothenic, perchloric, phenylacetic, phosphoric, propionic, pyroglutamic, L-pyroglutamic, D-pyroglutamic, saccharic, salicylic, 4-amino-salicylic, sebacic, stearic, succinic, sulfanilic, sulfuric, tannic, tartaric, D-tartaric, L-tartaric, thiocyanic, *p*-toluenesulfonic, trifluoroacetic, trifluoromethanesulfonic, undecylenic, and valeric acid. In one embodiment, the salt or pharmaceutically acceptable salt is formed from hydrochloric acid. In one embodiment, the salt or pharmaceutically acceptable salt is formed from sulfuric acid. In one embodiment, the salt or pharmaceutically acceptable salt is formed from methanesulfonic acid. In one embodiment, the salt or pharmaceutically acceptable salt is formed from

hydrobromic acid. In one embodiment, the salt or pharmaceutically acceptable salt is formed from acetic acid.

[0085] Suitable bases for use in the preparation of "salts" or "pharmaceutically acceptable salts", including, but not limited to, inorganic bases, such as magnesium hydroxide, calcium hydroxide, potassium hydroxide, zinc hydroxide, lithium hydroxide, or sodium hydroxide; and organic bases, such as primary, secondary, tertiary, and quaternary, aliphatic and aromatic amines, including L-arginine, benethamine, benzathine, choline, deanol, *N,N*-dicyclohexylmethyl amine, diethanolamine, diethylamine, dimethylamine, dipropylamine, diisopropylamine, diisopropylethyl amine, 2-(diethylamino)-ethanol, ethanolamine, ethylamine, ethylenediamine, isopropylamine, *N*-methyl-glucamine, hydrabamine, 1*H*-imidazole, L-lysine, 2,6-lutidine, morpholine, *N*-methyl-morpholine, 4-(2-hydroxyethyl)-morpholine, methylamine, piperidine, piperazine, propylamine, pyrrolidine, 1-(2-hydroxyethyl)-pyrrolidine, pyridine, quinuclidine, quinoline, isoquinoline, triethanolamine, trimethylamine, triethylamine, N-methyl-D-glucamine, 2-amino-2-(hydroxymethyl)-1,3-propanediol, and tromethamine.

[0086] In one embodiment, "salt," "salts" or "pharmaceutically acceptable salt" of 5-azacytidine refers to acid addition salt(s) of 5-azacytidine, derived from inorganic acids and/or organic acids, as described herein elsewhere. In some embodiments, the salt is formed from hydrochloric, hydrobromic, boric, phosphoric, or sulfuric acid. In one embodiment, the salt is formed from hydrochloric acid. In one embodiment, the salt is formed from sulfuric acid. In one embodiment, the salt is formed from methanesulfonic acid. In some embodiments, the salt is formed from acetic, citric, fumaric, maleic, malic, malonic, oxalic, succinic, tartaric, *p*-toluenesulfonic, trifluoromethanesulfonic, or trifluoroacetic acid.

[0087] As used herein, and unless otherwise specified, the term "solvate" refers to a compound provided herein or a salt thereof, which further includes a stoichiometric or non-stoichiometric amount of solvent bound by non-covalent intermolecular forces. Where the solvent is water, the solvate is a hydrate (*e.g.*, mono-hydrate, dihydrate, trihydrate, tetrahydrate, and the like).

[0088] As used herein, and unless otherwise indicated, the term "polymorph" refers to a solid crystalline form of a compound provided herein or a salt or complex thereof. Different polymorphs of the same compound can exhibit different physical, chemical, biological, and/or spectroscopic properties, among others.

[0089] As used herein, and unless otherwise specified, the term "stereoisomer" encompasses all enantiomerically/stereomerically pure and enantiomerically/ stereomerically enriched compounds provided herein.

[0090] As used herein and unless otherwise specified, the term "stereomerically pure" means a composition that comprises one stereoisomer of a compound and is substantially free of other stereoisomers of that compound. For example, a stereomerically pure composition of a compound having one chiral center will be substantially free of the opposite enantiomer of the compound. A stereomerically pure composition of a compound having two chiral centers will be substantially free of other diastereomers of the compound. A typical stereomerically pure compound comprises greater than about 80% by weight of one stereoisomer of the compound and less than about 20% by weight of other stereoisomers of the compound, greater than about 90% by weight of one stereoisomer of the compound and less than about 10% by weight of the other stereoisomers of the compound, greater than about 95% by weight of one stereoisomer of the compound and less than about 5% by weight of the other stereoisomers of the compound, greater than about 97% by weight of one stereoisomer of the compound and less than about 3% by weight of the other stereoisomers of the compound, or greater than about 99% by weight of one stereoisomer of the compound and less than about 1% by weight of the other stereoisomers of the compound.

[0091] As used herein and unless otherwise indicated, the term "stereomerically enriched" means a composition that comprises greater than about 55% by weight of one stereoisomer of a compound, greater than about 60% by weight of one stereoisomer of a compound, greater than about 70% by weight, or greater than about 80% by weight of one stereoisomer of a compound.

[0092] As used herein, and unless otherwise indicated, the term "enantiomerically pure" means a stereomerically pure composition of a compound having one chiral center. Similarly, the term "enantiomerically enriched" means a stereomerically enriched composition of a compound having one chiral center.

[0093] In certain embodiments, as used herein, and unless otherwise specified, "optically active" and "enantiomerically active" refer to a collection of molecules, which has an enantiomeric excess of no less than about 50%, no less than about 70%, no less than about 80%, no less than about 90%, no less than about 91%, no less than about 92%, no less than about 93%, no less than about 94%, no less than about 95%, no less than about 96%, no less than about 97%, no less than about 98%, no less than about 99%, no

less than about 99.5%, or no less than about 99.8%. In certain embodiments, the compound comprises about 95% or more of the desired enantiomer and about 5% or less of the less preferred enantiomer based on the total weight of the racemate in question.

[0094] As used herein, and unless otherwise specified, the term "racemic" or "racemate" refers to about 50% of one enantiomer and about 50% of the corresponding enantiomer relative to all chiral centers in the molecule.

[0095] In describing an optically active compound, the prefixes *R* and *S* are used to denote the absolute configuration of the molecule about its chiral center(s). The (+) and (–) are used to denote the optical rotation of the compound, that is, the direction in which a plane of polarized light is rotated by the optically active compound. The (–) prefix indicates that the compound is levorotatory, that is, the compound rotates the plane of polarized light to the left or counterclockwise. The (+) prefix indicates that the compound is dextrorotatory, that is, the compound rotates the plane of polarized light to the right or clockwise. However, the sign of optical rotation, (+) and (–), is not related to the absolute configuration of the molecule, *R* and *S*.

[0096] Unless otherwise specified, the compounds provided herein may be enantiomerically pure, such as a single enantiomer or a single diastereomer, or be stereoisomeric mixtures, such as a mixture of enantiomers, *e.g.*, a racemic mixture of two enantiomers; or a mixture of two or more diastereomers. Conventional techniques for the preparation/isolation of individual enantiomers include synthesis from a suitable optically pure precursor, asymmetric synthesis from achiral starting materials, or resolution of an enantiomeric mixture, for example, chiral chromatography, recrystallization, resolution, diastereomeric salt formation, or derivatization into diastereomeric adducts followed by separation.

[0097] It should be noted that where structural isomers are inter-convertible, the compound provided herein may exist as a single tautomer or a mixture of tautomers. This can take the form of proton tautomerism in the compound that contains, for example, an imino, keto, or oxime group; or so-called valence tautomerism in the compound that contain an aromatic moiety. It follows that a single compound may exhibit more than one type of isomerism.

[0098] As used herein, and unless otherwise indicated, the term "about" or "approximately" means an acceptable error for a particular value as determined by one of ordinary skill in the art, which depends in part on how the value is measured or determined. In certain embodiments, the term "about" or "approximately" means within

1, 2, 3, or 4 standard deviations. In certain embodiments, the term "about" or "approximately" means within 50%, 20%, 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, or 0.05% of a given value or range.

[0099] As used herein, and unless otherwise specified, a composition that is "substantially free" of a compound means that the composition contains less than about 20% by weight, less than about 10% by weight, less than about 5% by weight, less than about 3% by weight, less than about 1% by weight, less than about 0.1% by weight, less than about 0.01% by weight, less than about 0.001% by weight, or less than about 0.0001% by weight of the compound.

[00100] As used herein, and unless otherwise specified, a composition that is "substantially pure" means that the composition has a purity level of greater than about 80% by weight, greater than about 90% by weight, greater than about 95% by weight, greater than about 97% by weight, greater than about 99% by weight, greater than about 99.5% by weight, greater than about 99.9% by weight, greater than about 99.95% by weight, greater than about 99.99% by weight, greater than about 99.995% by weight, greater than about 99.999% by weight, greater than about 99.9995% by weight, or greater than about 99.9999% by weight.

[00101] As used herein, and unless otherwise specified, the term "pharmaceutically acceptable carrier," "pharmaceutically acceptable excipient," "physiologically acceptable carrier," or "physiologically acceptable excipient" refers to a pharmaceutically acceptable material, composition, or vehicle, such as a liquid or solid filler, diluent, solvent, or encapsulating material. In one embodiment, each component is "pharmaceutically acceptable" in the sense of being compatible with the other ingredients of a pharmaceutical formulation, and suitable for use in contact with the tissue or organ of humans and animals without excessive toxicity, irritation, allergic response, immunogenicity, or other problems or complications, commensurate with a reasonable benefit/risk ratio. *See, Remington: The Science and Practice of Pharmacy*, 21st Edition, Lippincott Williams & Wilkins (2005); *Handbook of Pharmaceutical Excipients*, 5th Edition, Rowe *et al.*, eds., The Pharmaceutical Press and the American Pharmaceutical Association (2005); and *Handbook of Pharmaceutical Additives*, 3rd Edition, Ash & Ash eds., Gower Publishing Company (2007); *Pharmaceutical Preformulation and Formulation*, 2nd Edition, Gibson ed., CRC Press (2009).

[00102] As used herein, and unless otherwise specified, the terms "active ingredient," "active substance," or "active pharmaceutical ingredient" refers to a compound or a

substance, which is administered, alone or in combination with other pharmaceutically active compound(s), and/or one or more pharmaceutically acceptable excipients, to a subject for treating, preventing, and/or ameliorating one or more symptoms of a condition, disorder, or disease. As used herein, "active ingredient," "active substance," and "active pharmaceutical ingredient" may be a pharmaceutically acceptable salt, solvate, hydrate, polymorph, or optically active isomer of a compound described herein.

[00103] As used herein, and unless otherwise specified, the terms "drug" and "therapeutic agent" refer to a compound, or a pharmaceutical composition thereof, which is administered to a subject for treating, preventing, managing, and/or ameliorating one or more symptoms of a condition, disorder, or disease.

[00104] As used herein, and unless otherwise indicated, the terms "treat," "treating" and "treatment" refer to the eradication or amelioration of a disease or disorder, or of one or more symptoms associated with the disease or disorder. In certain embodiments, the terms refer to minimizing the spread or worsening of the disease or disorder resulting from the administration of one or more prophylactic or therapeutic agents to a subject with such a disease or disorder. In some embodiments, the terms refer to the administration of a compound provided herein, with or without other additional active agent, after the onset of symptoms of the particular disease.

[00105] As used herein, and unless otherwise indicated, the terms "prevent," "preventing" and "prevention" refer to the prevention of the onset, recurrence or spread of a disease or disorder, or of one or more symptoms thereof. In certain embodiments, the terms refer to the treatment with or administration of a compound provided herein, with or without other additional active compound, prior to the onset of symptoms, particularly to patients at risk of disease or disorders provided herein. The terms encompass the inhibition or reduction of a symptom of the particular disease. Patients with familial history of a disease in particular are candidates for preventive regimens in certain embodiments. In addition, patients who have a history of recurring symptoms are also potential candidates for the prevention. In this regard, the term "prevention" may be interchangeably used with the term "prophylactic treatment."

[00106] As used herein, and unless otherwise specified, the terms "manage," "managing," and "management" refer to preventing or slowing the progression, spread or worsening of a disease or disorder, or of one or more symptoms thereof. Often, the beneficial effects that a subject derives from a prophylactic and/or therapeutic agent do not result in a cure of the disease or disorder. In this regard, the term "managing"

encompasses treating a patient who had suffered from the particular disease in an attempt to prevent or minimize the recurrence of the disease.

[00107] As used herein, and unless otherwise specified, the term "subject" is defined herein to include animals such as mammals, including, but not limited to, primates (*e.g.*, humans), cows, sheep, goats, horses, dogs, cats, rabbits, rats, mice, and the like. In specific embodiments, the subject is a human.

[00108] Unless otherwise specified, the compound provided herein may be provided as a prodrug, which is a functional derivative of the compound, for example, 5-azacytidine, and is readily convertible into the parent compound *in vivo*. Prodrugs are often useful because, in some situations, they may be easier to administer than the parent compound. They may, for instance, be bioavailable by oral administration whereas the parent compound is not. The prodrug may also have enhanced solubility in pharmaceutical compositions over the parent compound. A prodrug may be converted into the parent drug by various mechanisms, including enzymatic processes and metabolic hydrolysis. *See Prodrugs: Challenges and Rewards*, Valentino J. Stella *et al.*, eds., Springer Press, 2007; Harper, *Progress in Drug Research,* 1962, *4*, 221-294; Morozowich *et al.* in "Design of Biopharmaceutical Properties through Prodrugs and Analogs," Roche ed., APHA Acad. Pharm. Sci., 1977; "Bioreversible Carriers in Drug in Drug Design, Theory and Application," Roche ed., APHA Acad. Pharm. Sci., 1987; "Design of Prodrugs," Bundgaard, Elsevier, 1985; Wang *et al.*, *Curr. Pharm. Design,* 1999, *5*, 265-287; Pauletti *et al.*, *Adv. Drug. Delivery Rev.,* 1997, *27*, 235-256; Mizen *et al.*, *Pharm. Biotech.,* 1998, *11*, 345-365; Gaignault *et al.*, *Pract. Med. Chem.,* 1996, 671-696; Asgharnejad in "Transport Processes in Pharmaceutical Systems," Amidon *et al.*, ed., Marcell Dekker, 185-218, 2000; Balant *et al.*, *Eur. J. Drug Metab. Pharmacokinet.,* 1990, *15*, 143-53; Balimane and Sinko, *Adv. Drug Delivery Rev.,* 1999, *39*, 183-209; Browne, *Clin. Neuropharmacol.,* 1997, *20*, 1-12; Bundgaard, *Arch. Pharm. Chem.*, 1979, *86*, 1-39; Bundgaard, *Controlled Drug Delivery,* 1987, *17*, 179-96; Bundgaard, *Adv. Drug Delivery Rev.,* 1992, *8*, 1-38; Fleisher *et al.*, *Adv. Drug Delivery Rev.,* 1996, *19*, 115-130; Fleisher *et al.*, *Methods Enzymol.,* 1985, *112*, 360-381; Farquhar *et al.*, *J. Pharm. Sci.,* 1983, *72*, 324-325; Freeman *et al.*, *J. Chem. Soc., Chem. Commun.,* 1991, 875-877; Friis and Bundgaard, *Eur. J. Pharm. Sci.,* 1996, *4*, 49-59; Gangwar *et al.*, *Des. Biopharm. Prop. Prodrugs Analogs*, 1977, 409-421; Nathwani and Wood, *Drugs,* 1993, *45*, 866-94; Sinhababu and Thakker, *Adv. Drug Delivery Rev.,* 1996, *19*, 241-273; Stella *et al.*, *Drugs,* 1985, *29*, 455-73; Tan *et al.*, *Adv. Drug Delivery Rev.*, 1999, *39*, 117-151; Taylor, *Adv.*

Drug Delivery Rev., 1996, *19*, 131-148; Valentino and Borchardt, *Drug Discovery Today,* 1997, *2*, 148-155; Wiebe and Knaus, *Adv. Drug Delivery Rev.,* 1999, *39*, 63-80; and Waller *et al.*, *Br. J. Clin. Pharmac.,* 1989, *28*, 497-507.

[00109] Unless otherwise specified, the compounds described herein, including intermediates useful for the preparation of the compounds, which contain reactive functional groups (such as, without limitation, carboxy, hydroxy, and amino moieties), also encompass suitable protected derivatives thereof. "Protected derivatives" are those compounds in which a reactive site or sites are blocked with one or more protecting groups (also known as blocking groups). Suitable protecting groups for carboxy moieties include benzyl, *t*-butyl, and the like. Suitable protecting groups for amino and amido groups include acetyl, *t*-butyloxycarbonyl, benzyloxycarbonyl, silyl, and the like. Suitable protecting groups for hydroxy include benzyl, acetyl, silyl, and the like. Other suitable protecting groups are well known to those of ordinary skill in the art. The choice and use of protecting groups and the reaction conditions to install and remove protecting groups are described, for example, in T. W. Greene & P. G. M. Wuts, "Protective Groups in Organic Synthesis", Third Ed., John Wiley & Sons, Inc. 1999.

[00110] As used herein, and unless otherwise indicated, the term "process" refers to the methods disclosed herein which are useful for preparing a compound provided herein. Modifications to the methods disclosed herein (*e.g.*, starting materials, reagents, protecting groups, solvents, temperatures, reaction times, purification) that are well known to those of ordinary skill in the art are also encompassed by the present disclosure.

[00111] As used herein, and unless otherwise indicated, the term "adding," "reacting," "mixing," or the like means contacting one reactant, reagent, solvent, catalyst, reactive group, or the like with another reactant, reagent, solvent, catalyst, reactive group, or the like. Unless otherwise specified, reactants, reagents, solvents, catalysts, reactive group, or the like can be added individually, simultaneously, or separately, or can be added in any order. They can be added in the presence or absence of heat, and can optionally be added under an inert atmosphere. "Reacting" can refer to *in situ* formation or intra-molecular reaction where the reactive groups are in the same molecule.

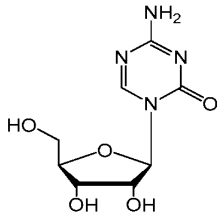
[00112] As used herein, and unless otherwise indicated, a reaction that is "substantially complete" or is driven to "substantial completion" means that the reaction contains more than about 50% by percent yield, more than about 60% by percent yield, more than about 70% by percent yield, more than about 80% by percent yield, more than about 90% by percent yield, more than about 95% by percent yield, or more than about 97% by percent

yield of the desired product. Alternatively, the terms "substantially complete" or "substantial completion" means that the reaction contains less than about 50% of a starting material relative to its starting amount, less than about 40%, less than about 30%, less than about 20%, less than about 10%, less than about 5%, less than about 3%, less than about 1%, less than about 0.5%, less than about 0.1%, less than about 0.05%, or less than about 0.01% of a starting material relative to its starting amount.

[00113] If there is a discrepancy between a depicted structure and a name given that structure, the depicted structure is to be accorded more weight. Furthermore, if the stereochemistry of a structure or a portion thereof is not indicated, *e.g.*, with bold or dashed lines, the structure or portion thereof is to be interpreted as encompassing all stereoisomers of it.

B. Processes

[00114] Provided herein are processes for the preparation of 5-azacytidine, or a salt, solvate, hydrate, or polymorph thereof. In general, the processes provided herein encompass safe, efficient, cost effective, and/or readily scaleable processes useful for the large scale or commercial production of 5-azacytidine, or a salt, solvate, hydrate, or polymorph thereof.



5-Azacytidine.

[00115] In one embodiment, provided herein are processes for the production of 5-azacytidine, or a salt, solvate, hydrate, or polymorph thereof, that is substantially pure. In one embodiment, provided herein are processes for the production of 5-azacytidine, or a salt, solvate, hydrate, or polymorph thereof, that is substantially chemically pure. In one embodiment, provided herein are processes for the production of 5-azacytidine, or a salt, solvate, hydrate, or polymorph thereof, that is substantially physically pure. In one embodiment, provided herein are processes for the production of 5-azacytidine, or a salt, solvate, hydrate, or polymorph thereof, suitable for use in humans, such as for treating, preventing, and/or managing diseases or conditions including cancer, disorders related to

abnormal cell proliferation, hematologic disorders, and myelodysplastic syndromes (MDS), among others.

[00116] In one embodiment, the processes provided herein encompass safe, cost-effective, and/or efficient means for the large scale or commercial production of 5-azacytidine, or a salt, solvate, hydrate, or polymorph thereof. In one embodiment, the processes provided herein produce 5-azacytidine, or a salt, solvate, hydrate, or polymorph thereof, on a scale of greater than 1 gram, greater than 10 gram, greater than 25 gram, greater than 50 gram, greater than 100 gram, greater than 250 gram, greater than 500 gram, greater than 1,000 gram, greater than 5,000 gram, greater than 10,000 gram, greater than 50,000 gram, or greater than 100,000 gram.

[00117] In one embodiment, the processes provided herein produce 5-azacytidine, or a salt, solvate, hydrate, or polymorph thereof, in an overall yield of greater than about 10%, greater than about 15%, greater than about 20%, greater than about 25%, greater than about 30%, greater than about 35%, greater than about 40%, greater than about 45%, greater than about 50%, greater than about 55%, greater than about 60%, greater than about 65%, greater than about 70%, greater than about 75%, greater than about 80%, greater than about 85%, greater than about 90%, or greater than about 95%, wherein the yield is calculated based on starting material, such as, *e.g.*, 5-azacytosine or a protected β-D-ribofuranose (*e.g.,* a compound of formula B).

[00118] In one embodiment, the processes provided herein produce 5-azacytidine, or a salt, solvate, hydrate, or polymorph thereof, that is substantially pure. In one embodiment, the purity of the 5-azacytidine, or a salt, solvate, hydrate, or polymorph thereof, is greater than about 95% w/w, greater than about 96% w/w, greater than about 97% w/w, greater than about 98% w/w, greater than about 99% w/w, greater than about 99.5% w/w, greater than about 99.8% w/w, greater than about 99.9% w/w, greater than about 99.95% w/w, greater than about 99.98% w/w, or greater than about 99.99% w/w relative to the total batch. In one embodiment, the total impurities in the 5-azacytidine, or a salt, solvate, hydrate, or polymorph thereof, is less than about 5% w/w, less than about 4% w/w, less than about 3% w/w, less than about 2% w/w, less than about 1% w/w, less than about 0.5% w/w, less than about 0.2% w/w, less than about 0.1% w/w, less than about 0.05% w/w, less than about 0.02% w/w, less than about 0.01% w/w, less than about 0.005% w/w, or less than about 0.001% w/w relative to the total batch. In one embodiment, an individual impurity component in the 5-azacytidine, or a salt, solvate, hydrate, or polymorph thereof, is less than about 5% w/w, less than about 2% w/w, less than about 1%

w/w, less than about 0.9% w/w, less than about 0.8% w/w, less than about 0.7% w/w, less than about 0.6% w/w, less than about 0.5% w/w, less than about 0.4% w/w, less than about 0.3% w/w, less than about 0.2% w/w, less than about 0.1% w/w, less than about 0.05% w/w, less than about 0.01% w/w, less than about 0.005% w/w, less than about 0.001% w/w, less than about 0.0005% w/w, or less than about 0.0001% w/w relative to the total batch. In one embodiment, the processes provided herein produce 5-azacytidine, or a salt, solvate, hydrate, or polymorph thereof, that is substantially physically and/or chemically pure.

[00119] In one embodiment, the processes provided herein produce 5-azacytidine, or a salt, solvate, hydrate, or polymorph thereof, that is substantially physically pure. In one embodiment, the processes provided herein produce a polymorph or a crystalline form of 5-azacytidine that is substantially physically pure. In one embodiment, the physical purity of the 5-azacytidine, or a salt, solvate, hydrate, or polymorph thereof, is greater than about 95% w/w, greater than about 96% w/w, greater than about 97% w/w, greater than about 98% w/w, greater than about 99% w/w, greater than about 99.5% w/w, greater than about 99.8% w/w, greater than about 99.9% w/w, greater than about 99.95% w/w, greater than about 99.98% w/w, or greater than about 99.99% w/w relative to the total batch. In one embodiment, the total impurities in the 5-azacytidine, or a salt, solvate, hydrate, or polymorph thereof, is less than about 5% w/w, less than about 4% w/w, less than about 3% w/w, less than about 2% w/w, less than about 1% w/w, less than about 0.5% w/w, less than about 0.2% w/w, less than about 0.1% w/w, less than about 0.05% w/w, less than about 0.02% w/w, less than about 0.01% w/w, less than about 0.005% w/w, or less than about 0.001% w/w relative to the total batch. In one embodiment, an individual impurity component in the 5-azacytidine, or a salt, solvate, hydrate, or polymorph thereof, is less than about 5% w/w, less than about 2% w/w, less than about 1% w/w, less than about 0.9% w/w, less than about 0.8% w/w, less than about 0.7% w/w, less than about 0.6% w/w, less than about 0.5% w/w, less than about 0.4% w/w, less than about 0.3% w/w, less than about 0.2% w/w, less than about 0.1% w/w, less than about 0.05% w/w, less than about 0.01% w/w, less than about 0.005% w/w, less than about 0.001% w/w, less than about 0.0005% w/w, or less than about 0.0001% w/w relative to the total batch.

[00120] In one embodiment, the processes provided herein produce 5-azacytidine, or a salt, solvate, hydrate, or polymorph thereof, that is substantially chemically pure. In one embodiment, the processes provided herein produce a polymorph or a crystalline form of 5-azacytidine that is substantially chemically pure. In one embodiment, the chemical

purity of the 5-azacytidine, or a salt, solvate, hydrate, or polymorph thereof, is greater than about 95% w/w, greater than about 96% w/w, greater than about 97% w/w, greater than about 98% w/w, greater than about 99% w/w, greater than about 99.5% w/w, greater than about 99.8% w/w, greater than about 99.9% w/w, greater than about 99.95% w/w, greater than about 99.98% w/w, or greater than about 99.99% w/w relative to the total batch. In one embodiment, the total impurities in the 5-azacytidine, or a salt, solvate, hydrate, or polymorph thereof, is less than about 5% w/w, less than about 4% w/w, less than about 3% w/w, less than about 2% w/w, less than about 1% w/w, less than about 0.5% w/w, less than about 0.2% w/w, less than about 0.1% w/w, less than about 0.05% w/w, less than about 0.02% w/w, less than about 0.01% w/w, less than about 0.005% w/w, or less than about 0.001% w/w relative to the total batch. In one embodiment, an individual impurity component in the 5-azacytidine, or a salt, solvate, hydrate, or polymorph thereof, is less than about 5% w/w, less than about 2% w/w, less than about 1% w/w, less than about 0.9% w/w, less than about 0.8% w/w, less than about 0.7% w/w, less than about 0.6% w/w, less than about 0.5% w/w, less than about 0.4% w/w, less than about 0.3% w/w, less than about 0.2% w/w, less than about 0.1% w/w, less than about 0.05% w/w, less than about 0.01% w/w, less than about 0.005% w/w, less than about 0.001% w/w, less than about 0.0005% w/w, or less than about 0.0001% w/w relative to the total batch.

[00121] In one embodiment, the impurity is detectable by HPLC (high performance liquid chromatography). In one embodiment, the impurity includes, but is not limited to, 6-amino-5-azacytosine, 2,4,6-triaminotriazine, 2,4-diaminotriazine, 6-methyl-5-azacytosine, 6-amino-5-azacytidine, 6-methyl-5-azacytidine, and 1-β-D-ribofuranosyl-3-guanylurea, among others. In one embodiment, the impurity is a metal based impurity, such as for example, impurities comprising tin or iron. In one embodiment, the impurity is a volatile organic compound, such as for example, methanol, dichloromethane, toluene, or triethylamine. In one embodiment, the impurity is an organic solvent, such as for example, methanol, dichloromethane, toluene or dimethylsulfoxide. In one embodiment, the weight loss on drying (LOD) of the 5-azacytidine, or a salt, solvate, hydrate, or polymorph thereof, is less than about 5% w/w, less than about 2% w/w, less than about 1% w/w, less than about 0.9% w/w, less than about 0.8% w/w, less than about 0.7% w/w, less than about 0.6% w/w, less than about 0.5% w/w, less than about 0.4% w/w, less than about 0.3% w/w, less than about 0.2% w/w, less than about 0.1% w/w, less than about 0.05% w/w, or less than about 0.01% w/w relative to the total batch.

[00122] In one embodiment, provided herein are processes for preparing 5-azacytidine, or a salt, solvate, hydrate, or polymorph thereof, comprising the steps of reacting a silylated 5-azacytosine with a protected β-D-ribofuranose in the presence of a metallic Lewis acid to yield a protected 5-azacytidine, deprotecting the protected 5-azacytidine to yield 5-azacytidine, and purifying the 5-azacytidine to yield 5-azacytidine, or a salt, solvate, hydrate, or polymorph thereof, that is substantially free of one or more impurities, including but not limited to, a metal-based impurity.

[00123] In one embodiment, the contemplated metal-based impurity comprises of the same metal element as that in the metallic Lewis acid, including but not limited to, tin, iron, zinc, titanium, aluminum, or boron, derived from metallic Lewis acid such as, stannic chloride, ferric chloride, zinc chloride, titanium tetrachloride, aluminum chloride, aluminum alkyl chloride, aluminum dialkyl chloride, aluminum trifluoride, or boron trifluoride, used in the preceding coupling reaction. In one embodiment, the total metal-based impurities in the 5-azacytidine, or a salt, solvate, hydrate, or polymorph thereof, is less than about 500 ppm (parts per million) w/w, less than about 200 ppm w/w, less than about 100 ppm w/w, less than about 50 ppm w/w, less than about 20 ppm w/w, less than about 10 ppm w/w, less than about 5 ppm w/w, less than about 2 ppm w/w, less than about 1 ppm w/w, less than about 0.5 ppm w/w, less than about 0.2 ppm w/w, or less than about 0.1 ppm w/w relative to the total batch. In one embodiment, an individual metal based impurity, such as for example, tin or iron content, in the 5-azacytidine, or a salt, solvate, hydrate, or polymorph thereof, is less than about 500 ppm w/w, less than about 200 ppm w/w, less than about 100 ppm w/w, less than about 50 ppm w/w, less than about 20 ppm w/w, less than about 10 ppm w/w, less than about 5 ppm w/w, less than about 2 ppm w/w, less than about 1 ppm w/w, less than about 0.5 ppm w/w, less than about 0.2 ppm w/w, less than about 0.1 ppm w/w, less than about 0.05 ppm w/w, less than about 0.02 ppm w/w, or less than about 0.01 ppm w/w relative to the total batch.

[00124] In specific embodiments, provided herein are processes for preparing 5-azacytidine, or a salt, solvate, hydrate, or polymorph thereof, comprising any one, two, three, four, five, or six of the steps of:

(a) reacting 5-azacytosine with a silylating reagent to yield a silylated 5-azacytosine;

(b) reacting the silylated 5-azacytosine with an acyl protected β-D-ribofuranose in the presence of a metallic Lewis acid; and quenching the reaction with water and at least one neutralizing reagent to yield a protected 5-azacytidine;

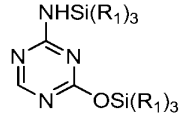
(c) reacting the protected 5-azacytidine with a base, selected from the group consisting of alkoxide, ammonia, and tetra-substituted ammonium hydroxide, in an alcohol to yield 5-azacytidine;

(d) contacting the 5-azacytidine from step (c) with an acid in an organic solvent to yield a salt of 5-azacytidine;

(e) contacting the salt of 5-azacytidine from step (d) with a base in an organic solvent to yield 5-azacytidine as a free base; and

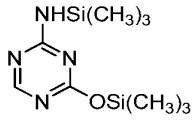
(f) re-crystallizing the 5-azacytidine from step (e).

[00125] In one embodiment, the silylated 5-azacytosine is a compound of formula (A):



(A),

wherein each R_1 is independently optionally substituted C_1–C_{10} alkyl, optionally substituted C_3–C_{10} cycloalkyl, or optionally substituted C_6–C_{10} aryl. In some embodiments, R_1 is straight chain alkyl, branched alkyl, cycloalkyl, or aryl, including but not limited to, methyl, ethyl, *i*-propyl, *t*-butyl, phenyl, xylyl, and benzyl. In some embodiments, R_1 is methyl. In some embodiments, R_1 is ethyl. In some embodiments, R_1 is isopropyl. In some embodiments, the silylated 5-azacytosine is:



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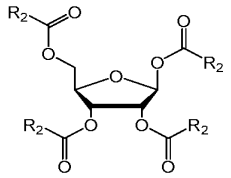
[00126] A variety of suitable protecting groups may be used to protect the hydroxyl groups in a β-D-ribofuranose to form a protected β-D-ribofuranose, which may be coupled with a silylated 5-azacytosine (*e.g.*, a compound of formula (A)). The protecting groups may be removed under suitable conditions at a later stage of the reaction sequence (*e.g.,* after the protected 5-azacytidine is formed). One skilled in the art will recognize and select suitable protecting groups for the hydroxyl moieties of β-D-ribofuranose, and will select appropriate conditions for installing such protecting groups. *See, e.g.*, T. W. Greene, & P. G. M. Wuts, "Protective Groups in Organic Synthesis", Third Ed., John Wiley & Sons, Inc., 1999. In one embodiment, the protected β-D-ribofuranose is purchased from a commercial source or prepared following a literature procedure. In one embodiment, the protected β-D-ribofuranose has one or more acyl, alkyl, silyl, acetal, or

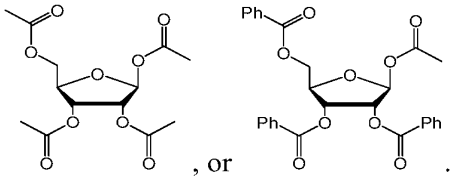
ketal protecting groups, or a combination thereof. In one embodiment, the protected β-D-ribofuranose has one, two, three, or four of the hydroxyl groups protected by suitable protecting group(s). In one embodiment, all four hydroxyl groups are protected in the protected β-D-ribofuranose. In one embodiment, the protected β-D-ribofuranose is acyl protected β-D-ribofuranose. In one embodiment, the protected β-D-ribofuranose is tetra-acyl protected β-D-ribofuranose.

[00127] In one embodiment, the protected β-D-ribofuranose is a compound of formula (B):



(B),

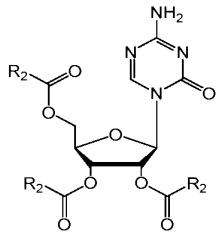
wherein each R_2 is independently hydrogen, optionally substituted C_1–C_{10} alkyl, optionally substituted C_3–C_{10} cycloalkyl, or optionally substituted C_6–C_{10} aryl. In some embodiments, R_2 is optionally substituted methyl or optionally substituted phenyl. In some embodiments, R_2 is methyl. In some embodiments, R_2 is phenyl. In some embodiments, the protected β-D-ribofuranose is:

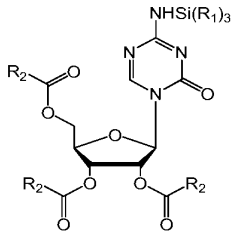


, or .

[00128] In one embodiment, the metallic Lewis acid is a Lewis acid that contains a metal atom, including, but not limited to, tin, iron, zinc, titanium, aluminum, and boron. In one embodiment, the metallic Lewis acid is selected from the group consisting of stannic chloride, ferric chloride, zinc chloride, titanium tetrachloride, aluminum chloride, aluminum alkyl chloride (*e.g.*, $EtAlCl_2$), aluminum dialkyl chloride (*e.g.*, Et_2AlCl), aluminum fluoride, boron trifluoride, and the like. In one embodiment, the metallic Lewis acid is stannic chloride or ferric chloride. In one embodiment, the metallic Lewis acid is stannic chloride. In one embodiment, the metallic Lewis acid is ferric chloride.

[00129] In one embodiment, the protected 5-azacytidine is a compound of formula (C) or (D):


(C),

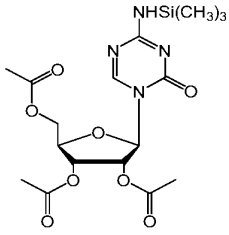

(D),

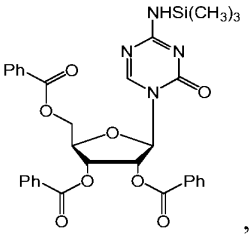
wherein each R_1 is independently optionally substituted C_1–C_{10} alkyl, optionally substituted C_3–C_{10} cycloalkyl, or optionally substituted C_6–C_{10} aryl; and each R_2 is independently hydrogen, optionally substituted C_1–C_{10} alkyl, optionally substituted C_3–C_{10} cycloalkyl, or optionally substituted C_6–C_{10} aryl. In some embodiments, R_1 is straight chain alkyl, branched alkyl, cycloalkyl, or aryl, including but not limited to, methyl, ethyl, *i*-propyl, *t*-butyl, phenyl, xylyl, and benzyl. In some embodiments, R_1 is methyl. In some embodiments, R_2 is optionally substituted methyl or optionally substituted phenyl. In some embodiments, R_2 is methyl. In some embodiments, R_2 is phenyl.

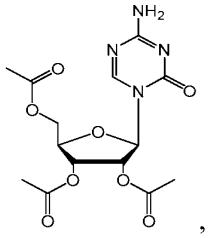
[00130] In one embodiment, the protected 5-azacytidine is a compound of formula (C).

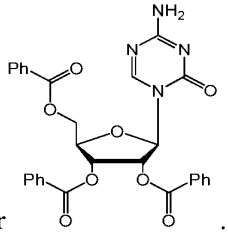
[00131] In one embodiment, a compound of formula (D) is formed from the coupling reaction between a silylated 5-azacytosine and a protected β-D-ribofuranose, and then converted to a compound of formula (C) during the aqueous work-up of the reaction. In one embodiment, a compound of formula (C) is isolated after the aqueous work-up of the reaction.

[00132] In specific embodiments, the protected 5-azacytidine is:

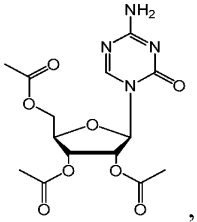

,

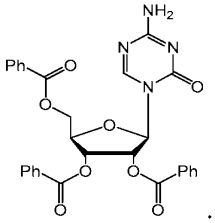

,


, or

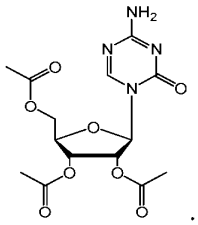

.

[00133] In specific embodiments, the protected 5-azacytidine is:

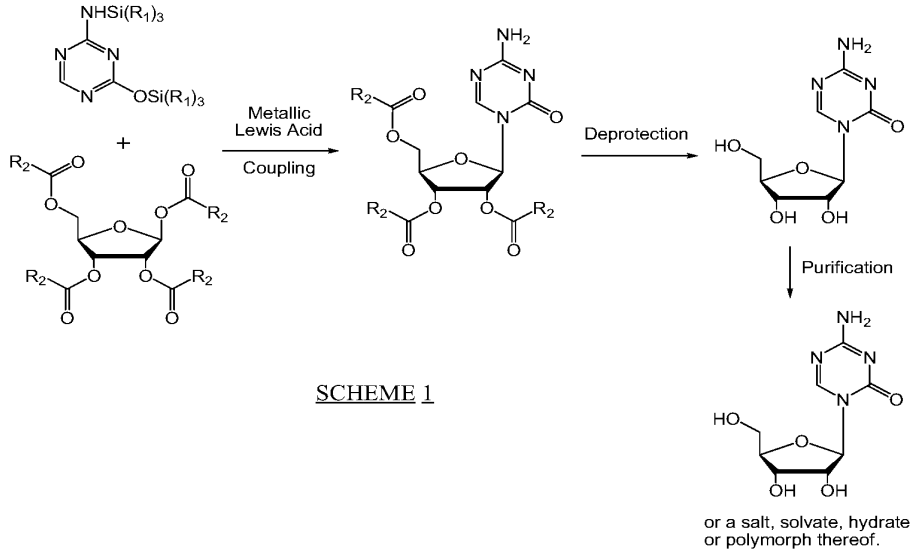

, or


.

[00134] In one embodiment, the protected 5-azacytidine is:



[00135] In one embodiment, provided herein are processes for preparing 5-azacytidine, or a salt, solvate, hydrate, or polymorph thereof, as described in Scheme 1 below:



SCHEME 1

wherein R_1 and R_2 are defined herein elsewhere.

[00136] In one embodiment, purification of 5-azacytidine encompasses the formation of a salt of 5-azacytidine to substantially reduce the impurity content, such as, *e.g.*, a metal-based impurity, in a given batch of 5-azacytidine. In one embodiment, an acid addition salt of 5-azacytidine is formed to substantially reduce the impurity content, such as, *e.g.*, a metal-based impurity, in a given batch of 5-azacytidine. For example, in specific embodiments, 5-azacytidine is stirred with an acid, such as hydrochloric acid, in a solvent, such as an alcohol, for example, methanol. The acid addition salt of 5-azacytidine is produced. The salt of 5-azacytidine is then isolated, such as for example, collected by filtration, and washed with a solvent, such as an alcohol, for example, methanol. Optionally, the salt of 5-azacytidine is dried under vacuum at ambient temperature or at elevated temperature, such as 50–60˚C. The salt of 5-azacytidine may be isolated as a

crystalline solid, which has a substantially reduced impurity content, such as metal content, for example, tin or iron content. In one embodiment, the salt of 5-azacytidine is substantially physically pure. In one embodiment, the salt of 5-azacytidine is substantially chemically pure.

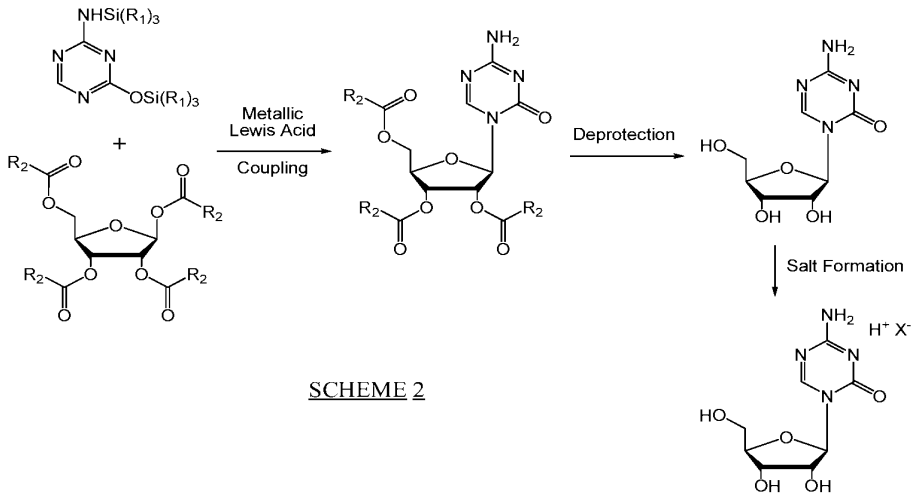
[00137] In one embodiment, the salt of 5-azacytidine is treated with a base to break the acid addition salt of 5-azacytidine and to form the free base of 5-azacytidine. In some embodiments, about 1.0, about 1.1, about 1.2, about 1.3, about 1.4, about 1.5, about 1.6, about 1.7, about 1.8, about 1.9, about 2.0, about 2.5, or about 3.0 equivalent of the base is used to generate the free base of 5-azacytidine from the acid addition salt. In other embodiments, greater than about 3.0 equivalent of the base is used to generate the free base of 5-azacytidine from the acid addition salt. In specific embodiments, for example, the salt of 5-azacytidine is stirred with a base, such as, an organic base, for example, triethylamine, in a solvent, such as an alcohol, for example, methanol. The free base of 5-azacytidine is formed. The free base of 5-azacytidine is then isolated, such as for example, collected by filtration, and washed with a solvent, such as an alcohol, for example, methanol. Washing may be continued until the filtrate is substantially free of the acidic salt counter ion, such as chloride. Optionally, the isolated free base of 5-azacytidine is dried under vacuum at ambient temperature or at elevated temperature, such as 50–60˚C. The free base of 5-azacytidine may be isolated as a crystalline solid, which has a substantially reduced impurity content, such as metal content, for example, tin or iron content. In one embodiment, the free base of 5-azacytidine is substantially physically pure. In one embodiment, the free base of 5-azacytidine is substantially chemically pure.

[00138] In one embodiment, 5-azacytidine is purified by re-crystallization. For example, in specific embodiments, 5-azacytidine or a salt thereof is dissolved in a solvent, such as dimethylsulfoxide (DMSO), at a temperature sufficient to allow the 5-azacytidine to dissolve, such as a temperature of greater than about 85˚C. Optionally, the solution of 5-azacytidine is filtered, for example, through filter paper. The filtration may be performed at an elevated temperature, such as a temperature of greater than about 85˚C. Optionally, hot DMSO may be used to wash the particles retained by filtration. To the solution of 5-azacytidine is added an anti-solvent, such as, *e.g.,* an alcohol, for example, methanol. The mixture is cooled, and 5-azacytidine re-crystallizes. In other embodiments, the anti-solvent may be added during the cooling step of the DMSO solution of 5-azacytidine. For example, the hot DMSO solution of 5-azacytidine may be first cooled to a certain temperature, the anti-solvent is then added, followed by further

cooling of the resulting mixture. The crystalline 5-azacytidine may be collected by filtration. The crystalline 5-azacytidine may be washed with a solvent, such as, *e.g.*, an alcohol, for example, methanol. In some embodiments, the re-crystallized 5-azacytidine is dried under vacuum at ambient temperature or at elevated temperature, such as about 40˚C, about 50˚C, about 60˚C, about 70˚C, about 80˚C, or about 90˚C. The re-crystallized 5-azacytidine may be isolated as a crystalline solid, which is substantially free of impurity content, such as metal content, for example, tin or iron content. In one embodiment, the re-crystallized 5-azacytidine is substantially physically pure. In one embodiment, the re-crystallized 5-azacytidine is substantially chemically pure.

[00139] In one embodiment, purification of 5-azacytidine comprises a combination of the salt formation step, the free base formation step, and/or the re-crystallization step. In one embodiment, purification of 5-azacytidine comprises the steps of (1) salt formation, (2) free base formation, and (3) re-crystallization. In another embodiment, purification of 5-azacytidine comprises the steps of (1) salt formation, (2) re-crystallization, and (3) free base formation. In another embodiment, purification of 5-azacytidine comprises the steps of (1) re-crystallization, (2) salt formation, and (3) free base formation. In another embodiment, purification of 5-azacytidine comprises the steps of (1) salt formation, and (2) free base formation. In another embodiment, purification of 5-azacytidine comprises the steps of (1) salt formation, and (2) re-crystallization. In another embodiment, purification of 5-azacytidine comprises the steps of (1) re-crystallization, and (2) salt formation.

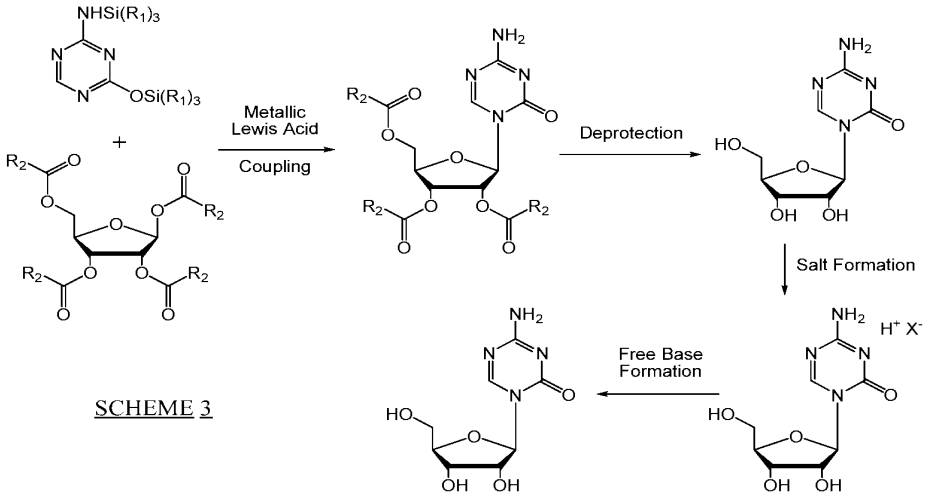
[00140] In one embodiment, provided herein are processes for preparing 5-azacytidine, or a salt, solvate, hydrate, or polymorph thereof, as described in Scheme 2 below:



SCHEME 2

wherein R_1 and R_2 are defined herein elsewhere, X^- is one or more salt counter ion(s), as defined herein elsewhere.

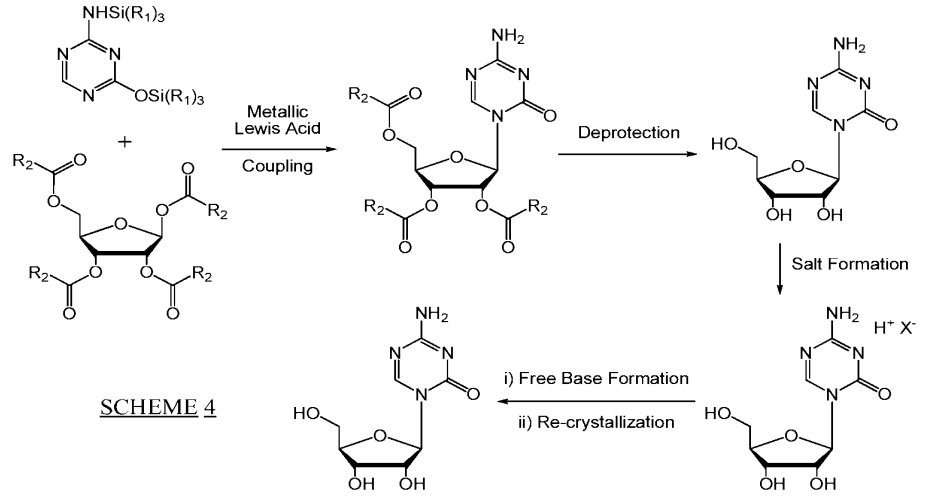
[00141] In one embodiment, provided herein are processes for preparing 5-azacytidine, or a salt, solvate, hydrate, or polymorph thereof, as described in Scheme 3 below:



SCHEME 3

wherein R_1 and R_2 are defined herein elsewhere, X^- is one or more salt counter ion(s), as defined herein elsewhere.

[00142] In one embodiment, provided herein are processes for preparing 5-azacytidine, or a salt, solvate, hydrate, or polymorph thereof, as described in Scheme 4 below:



SCHEME 4

wherein R_1 and R_2 are defined herein elsewhere, X^- is one or more salt counter ion(s), as defined herein elsewhere.

[00143] In one embodiment, provided herein are processes for preparing 5-azacytidine, or a salt, solvate, hydrate, or polymorph thereof, comprising any one, two, three, four, five, or six of the steps of:

(a) reacting 5-azacytosine with a silylating reagent to yield a silylated 5-azacytosine;

(b) reacting the silylated 5-azacytosine with an acyl protected β-D-ribofuranose in the presence of a metallic Lewis acid; and quenching the reaction with water and at least one neutralizing reagent to yield a protected 5-azacytidine;

(c) reacting the protected 5-azacytidine with a base, selected from the group consisting of alkoxide, ammonia, and tetra-substituted ammonium hydroxide, in an alcohol to yield 5-azacytidine;

(d) contacting the 5-azacytidine from step (c) with an acid in an organic solvent to yield a salt of 5-azacytidine;

(e) contacting the salt of 5-azacytidine from step (d) with a base in an organic solvent to yield 5-azacytidine as a free base; and

(f) re-crystallizing the 5-azacytidine from step (e).

1. Step (a) — Silylation of 5-Azacytosine

[00144] In one embodiment, the silylating reagent used in step (a) is a trimethylsilyl (TMS) reagent (*i.e.* R_1 is methyl). In one embodiment, the silylating reagent used in step (a) is a mixture of two or more TMS reagents. In one embodiment, the silylation reagent used in step (a) is selected from the group consisting of hexamethyldisilazane (HMDS) and chlorotrimethylsilane (TMSCl). In some embodiments, the silylating reagent comprises of a mixture of HMDS and TMSCl. In some embodiments, the silylating reagent comprises HMDS. In some embodiments, the silylating reagent is HMDS.

[00145] In one embodiment, the silylating reagent used in step (a) is in molar excess relative to 5-azacytosine. In one embodiment, the silylation reaction of step (a) uses excess HMDS. In one embodiment, the silylation reaction of step (a) uses an excess of about 7-volume of HMDS (*e.g.*, 700 mL of HMDS relative to 100 g of 5-azacytosine). In one embodiment, the silylation reaction of step (a) uses an excess of about 4-volume, about 5-volume, about 7-volume, about 10-volume, about 12-volume, or about 14-volume, of HMDS, relative to 5-azacytosine.

[00146] In one embodiment, the silylation reaction of step (a) is carried out in the presence of a catalyst. In one embodiment, the catalyst is ammonium sulfate.

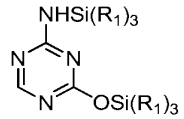
[00147] In one embodiment, the silylation reaction is carried out at room temperature. In one embodiment, the silylation reaction is carried out at elevated temperature. In one embodiment, the silylation reaction is carried out at a temperature of greater than about 50˚C, greater than about 60˚C, greater than about 70˚C, greater than about 80˚C, greater than about 90˚C, greater than about 100˚C, greater than about 110˚C, greater than about 120˚C, greater than about 130˚C, greater than about 140˚C, greater than about 150˚C, greater than about 160˚C, greater than about 170˚C. In one embodiment, the silylation reaction is carried out at a temperature of between about 50˚C and about 170˚C, between about 60˚C and about 165˚C, between about 70˚C and about 160˚C, between about 80˚C and about 155˚C, between about 90˚C and about 150˚C, between about 100˚C and about 145˚C, between about 110˚C and about 140˚C, between about 120˚C and about 140˚C. In one embodiment, the reaction is carried out at a temperature of about 125˚C. In one embodiment, the reaction is carried out at a temperature of about 130˚C.

[00148] In one embodiment, the silylation reaction is carried out under an inert atmosphere. In one embodiment, the silylation reaction is carried out under nitrogen. In one embodiment, the silylation reaction is carried out under argon.

[00149] The reaction time of the silylation reaction can vary from about 0.5 hr to about 24 hr, depending on the reaction temperature, the silylating reagent used, and the concentration of reagents in the reaction mixture. In general, the higher the reaction temperature, the shorter the reaction time. In one embodiment, the reaction time is about 0.5 hr, about 1 hr, about 2 hr, about 3 hr, about 4 hr, about 5 hr, about 6 hr, about 7 hr, about 8 hr, about 9 hr, about 10 hr, about 12 hr, about 14 hr, about 16 hr, about 18 hr, about 20 hr, about 22 hr, or about 24 hr. In one embodiment, the reaction time is about 2 hr, about 3 hr, about 4 hr, about 5 hr, or about 6 hr, at a reaction temperature of about 125˚C, in the presence of a catalyst, such as ammonium sulfate, when the silylating reagent comprises HMDS. In some embodiments, the reaction mixture becomes a clear solution when the reaction is substantially complete.

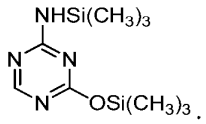
[00150] In one embodiment, the silylation reaction is carried out in the absence of a solvent using a molar excess of silylating reagent, and optionally in the presence of a catalyst. In one embodiment, the silylation reaction is carried out in the presence of a solvent. In one embodiment, the silylation reaction is carried out in the presence of a polar organic solvent, such as for example, acetonitrile. In one embodiment, the solvent is removed under vacuum (*e.g.*, 10–15 mmHg) after the completion of the reaction.

[00151] In one embodiment, the silylated 5-azacytosine is a compound of formula (A):



(A),

wherein R_1 is defined herein elsewhere. In one embodiment, the silylated 5-azacytosine is:



[00152] In one embodiment, the silylated 5-azacytosine is isolated as a solid. In other embodiments, the silylated 5-azacytosine is used directly in step (b) without isolation. In some embodiments, the silylated 5-azacytosine is isolated under an inert atmosphere, such as nitrogen and/or argon. In one embodiment, the silylated 5-azacytosine is isolated by removing the silylating reagents using vacuum distillation. In one embodiment, the silylated 5-azacytosine is isolated by filtration.

[00153] In one embodiment, the silylated 5-azacytidine is prepared by heating a suspension of 5-azacytosine, one or more TMS reagents (present in an excess molar ratio over the 5-azacytosine) and a catalyst, such as for example, ammonium sulfate, at reflux without a solvent until a clear solution results, wherein trimethylsilylated 5-azacytidine (*i.e.* a compound of formula (A) wherein R_1 is methyl) is formed. In some embodiments, the TMS reagent is HMDS.

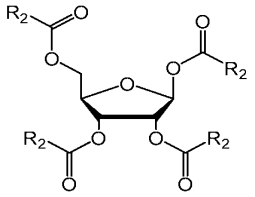
[00154] In one embodiment, the silylated 5-azacytosine is isolated by techniques known in the art. In one embodiment, the isolated silylated 5-azacytosine is used with or without drying in the subsequent coupling reaction with the protected β-D-ribofuranose. In one embodiment, the silylation reaction mixture is cooled to ambient temperature, where the silylated 5-azacytosine crystallizes from the reaction mixture. In one embodiment, the crystallization of silylated 5-azacytosine is facilitated by a suitable anti-solvent, such as for example, heptane. In one embodiment, the silylated 5-azacytosine is isolated by filtration under inert atmosphere. In one embodiment, the silylated 5-azacytosine is washed with a suitable washing solvent, such as for example, heptane. In another embodiment, the silylated 5-azacytosine is isolated as a solid residue by removing excess TMS reagent and any solvent (if present) by vacuum distillation. In one embodiment, a suitable solvent, such as for example, toluene, is added to the solid residue of silylated 5-azacytosine, and

the solvent is removed by vacuum distillation, wherein any residual TMS reagent, such as HMDS, is removed together with the solvent. In one embodiment, the excess HMDS is recovered by vacuum distillation and may be reused as a silylating reagent. In one embodiment, the isolated silylated 5-azacytosine is dissolved in a suitable solvent, such as for example, dichloromethane, acetonitrile, or 1,2-dichloroethane, for use in the subsequent coupling step.

[00155] In one embodiment, the silylated 5-azacytosine is prepared "in situ" from 5-azacytosine and an equivalent molar amount of silylating reagent(s) (such as a mixture of HMDS and TMSCl) in a suitable solvent in the presence or absence of a catalyst at reflux. In one embodiment, the solvent is a dry organic solvent. In one embodiment, the solvent is a dry non-polar organic solvent, including but not limited to, a halogenated solvent. In one embodiment, the solvent is a dry polar organic solvent, including but not limited to, acetonitrile. The resulting silylated 5-azacytosine can be used directly in the subsequent coupling step without isolation.

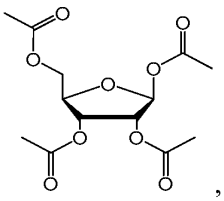
2. Step (b) — Coupling

[00156] In one embodiment, the acyl protected β-D-ribofuranose used in step (b) is a compound of formula (B):

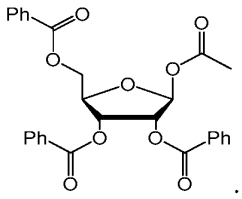


(B),

wherein R_2 is defined herein elsewhere. In one embodiment, the acyl protected β-D-ribofuranose is:



, or



.

[00157] In one embodiment, the acyl protected β-D-ribofuranose used in step (b) may be purchased from a commercial supplier (*e.g.*, Suven Life Sciences Limited). In one

embodiment, the acyl protected β-D-ribofuranose used in step (b) may be prepared from β-D-ribofuranose following literature procedures.

[00158] In one embodiment, the metallic Lewis acid used in step (b) is a Lewis acid that contains a metal atom, such as, *e.g.*, tin, iron, zinc, titanium, aluminum, and boron. In one embodiment, the metallic Lewis acid is selected from the group consisting of stannic chloride, ferric chloride, zinc chloride, titanium tetrachloride, aluminum chloride, aluminum alkyl chloride (*e.g.*, $EtAlCl_2$), aluminum dialkyl chloride (*e.g.*, Et_2AlCl), aluminum fluoride, boron trifluoride, and the like. In one embodiment, the metallic Lewis acid is stannic chloride or ferric chloride. In one embodiment, the metallic Lewis acid is stannic chloride. In one embodiment, the metallic Lewis acid is ferric chloride.

[00159] In one embodiment, the reaction of step (b) is carried out in a dry organic solvent. In one embodiment, the reaction of step (b) is carried out in a solvent with low water solubility. In one embodiment, the reaction of step (b) is carried out in a dry organic non-polar solvent with low water solubility. In one embodiment, the reaction of step (b) is carried out in a halogenated solvent, including but not limited to, dichloromethane, carbon tetrachloride, chloroform, and dichloroethane. In one embodiment, the reaction of step (b) is carried out in dichloromethane. In one embodiment, the reaction of step (b) is carried out in a dry polar organic solvent. In one embodiment, the reaction of step (b) is carried out in acetonitrile.

[00160] In one embodiment, the reaction of step (b) is carried out at a temperature of less than about 30˚C. In one embodiment, the reaction of step (b) is carried out at a temperature of less than about 25˚C. In one embodiment, the reaction of step (b) is carried out at a temperature of less than about 20˚C. In one embodiment, the reaction of step (b) is carried out at a temperature of less than about 15˚C. In one embodiment, the reaction of step (b) is carried out at a temperature of less than about 10˚C. In one embodiment, the reaction of step (b) is carried out at a temperature of less than about 5˚C. In one embodiment, the reaction of step (b) is carried out at a temperature of less than about 0˚C. In one embodiment, the reaction of step (b) is carried out at a temperature of greater than about –20˚C. In one embodiment, the reaction of step (b) is carried out at a temperature of greater than about –10˚C. In one embodiment, the reaction of step (b) is carried out at a temperature of greater than about 0˚C. In one embodiment, the reaction of step (b) is carried out at a temperature of between about 0˚C and about 5˚C. In one embodiment, the reaction of step (b) is carried out at a temperature of between about –5˚C and about –10˚C.

In one embodiment, the reaction of step (b) is carried out at a temperature of between about –15˚C and about –20˚C.

[00161] In one embodiment, the coupling reaction of step (b) is carried out under an inert atmosphere. In one embodiment, the coupling reaction is carried out under nitrogen. In one embodiment, the coupling reaction is carried out under argon.

[00162] The reaction time of the coupling reaction of step (b) can vary from about 1 hr to about 24 hr, depending on the reaction temperature, the reagents used, and the concentration of reagents in the reaction mixture. In general, the higher the reaction temperature, the shorter the reaction time, however, higher reaction temperature may give rise to side reactions or decomposition of the product. In specific embodiments, the reaction time of step (b) is about 1 hr, about 2 hr, about 3 hr, about 4 hr, about 5 hr, about 6 hr, about 7 hr, about 8 hr, about 9 hr, about 10 hr, about 12 hr, about 14 hr, about 16 hr, about 18 hr, about 20 hr, about 22 hr, or about 24 hr. In certain embodiments, the reaction time is about 2 hr, about 3 hr, about 4 hr, about 5 hr, about 6 hr, about 7 hr, about 8 hr, about 9 hr, or about 10 hr, at a reaction temperature of between about 0˚C and about 5˚C, when the Lewis acid is stannic chloride. In certain embodiments, the reaction time is about 2 hr, about 4 hr, about 6 hr, about 8 hr, about 10 hr, about 12 hr, about 14 hr, about 16 hr, about 18 hr, about 20 hr, about 22 hr, or about 24 hr, at a reaction temperature of between about 0˚C and about 5˚C, when the Lewis acid is ferric chloride. In some embodiments, the progress of the reaction is monitored, such as by taking an aliquot of the reaction mixture, quenching it with an aqueous solution, and passing it through HPLC. In one embodiment, the reaction is quenched when the reaction is determined to be substantially complete, *e.g.*, via reaction progress monitoring.

[00163] In one embodiment, the reaction of step (b) is performed by stirring the silylated 5-azacytosine with the acyl protected β-D-ribofuranose in a solvent maintained at a temperature of between about 0˚C and about 5˚C; adding the metallic Lewis acid, such as stannic chloride or ferric chloride, while maintaining the temperature of the reaction mixture; and stirring the reaction mixture at a temperature of between about 0˚C and about 5˚C until the reaction is substantially complete. In one embodiment, the reaction of step (b) is performed by stirring the acyl protected β-D-ribofuranose and the metallic Lewis acid, such as stannic chloride or ferric chloride, in a solvent maintained at a temperature of between about 0˚C and about 5˚C; adding the silylated 5-azacytosine while maintaining the temperature of the reactions mixture; and stirring the reaction mixture at a temperature of between about 0˚C and about 5˚C until the reaction is substantially complete.

[00164] In one embodiment, the molar ratio of the acyl protected β-D-ribofuranose used in the reaction of step (b) relative to 5-azacytosine used in step (a) is about 0.5 (*i.e.*, [acyl protected β-D-ribofuranose] / [5-azacytosine] = 0.5), about 0.6, about 0.7, about 0.8, about 0.9, about 1.0, about 1.1, about 1.2, about 1.3, about 1.4, about 1.5, about 1.6, about 1.7, about 1.8, about 1.9, or about 2.0. In one embodiment, the molar ratio of the acyl protected β-D-ribofuranose used in the reaction of step (b) relative to 5-azacytosine used in step (a) is about 0.9. In one embodiment, the molar ratio of the acyl protected β-D-ribofuranose used in the reaction of step (b) relative to 5-azacytosine used in step (a) is about 1.0. In one embodiment, the molar ratio of the acyl protected β-D-ribofuranose used in the reaction of step (b) relative to 5-azacytosine used in step (a) is about 1.1.

[00165] In one embodiment, the molar ratio of the metallic Lewis acid used in the reaction of step (b) relative to 5-azacytosine used in step (a) is about 0.5 (*i.e.*, [Lewis acid] / [5-azacytosine] = 0.5), about 0.6, about 0.7, about 0.8, about 0.9, about 1.0, about 1.1, about 1.2, about 1.3, about 1.4, about 1.5, about 1.6, about 1.7, about 1.8, about 1.9, about 2.0, about 2.5, about 3.0, or greater than about 3.0. In one embodiment, the molar ratio of the metallic Lewis acid used in the reaction of step (b) relative to 5-azacytosine used in step (a) is about 0.9. In one embodiment, the molar ratio of the metallic Lewis acid used in the reaction of step (b) relative to 5-azacytosine used in step (a) is about 1.0. In one embodiment, the molar ratio of the metallic Lewis acid used in the reaction of step (b) relative to 5-azacytosine used in step (a) is about 1.1. In one embodiment, the molar ratio of the metallic Lewis acid used in the reaction of step (b) relative to 5-azacytosine used in step (a) is about 1.5.

[00166] In one embodiment, the reaction of step (b) is quenched with water and one or more neutralizing reagents(s) to yield a quenched composition. In one embodiment, the reaction of step (b) is quenched at a temperature of about 15°C, about 10°C, about 5°C, or about 0°C. In one embodiment, the reaction of step (b) is quenched at a temperature of less than about 10°C. The quenching step may be exothermic. In one embodiment, the reaction mixture is cooled in a cooling bath. In one embodiment, water and one or more neutralizing reagent(s) are added slowly to the reaction mixture of step (b) to maintain the temperature of the reaction mixture. In one embodiment, water and the neutralizing reagent(s) are added together as a solution to the reaction mixture of step (b). In one embodiment, water and the neutralizing reagent(s) are added separately and sequentially. In one embodiment, water is chilled before addition to the reaction mixture of step (b). In one embodiment, an organic solvent is added to the reaction mixture of step (b) when the

reaction is quenched, such organic solvent may be the same solvent used in the reaction, such as, for example, dichloromethane.

[00167] In one embodiment, the neutralizing reagent in step (b) is an inorganic reagent. In one embodiment, the neutralizing reagent in step (b) is an inorganic base, such as, for example, lithium hydroxide, sodium hydroxide, and potassium hydroxide. In one embodiment, the neutralizing reagent in step (b) is an inorganic salt, such as, for example, sodium carbonate, sodium bicarbonate, potassium carbonate, potassium bicarbonate, lithium carbonate, lithium bicarbonate, sodium phosphate, sodium hydrogenphosphate, sodium dihydrogenphosphate, potassium phosphate, potassium hydrogenphosphate, potassium dihydrogenphosphate, lithium phosphate, lithium hydrogenphosphate, lithium dihydrogenphosphate, sodium citrate, potassium citrate, and lithium citrate. In one embodiment, the neutralizing reagent in step (b) is a carbonate or bicarbonate salt, or a mixture thereof. In one embodiment, the neutralizing reagent in step (b) is sodium carbonate or sodium bicarbonate, or a mixture thereof. In one embodiment, the neutralizing reagent in step (b) is a mixture of sodium carbonate and sodium bicarbonate in 1:1 molar ratio. In one embodiment, the neutralizing reagent in step (b) is a mixture of sodium carbonate and sodium bicarbonate in 1:1 weight ratio. In one embodiment, the neutralizing reagent in step (b) is sodium carbonate. In one embodiment, the neutralizing reagent in step (b) is sodium bicarbonate. In one embodiment, the neutralizing reagent is first dissolved in water and the solution is added to the reaction mixture of step (b). In one embodiment, the neutralizing reagent is added to the reaction mixture of step (b) as a solid. In one embodiment, the neutralizing reagent is added first to the reaction mixture of step (b), and then water is added to the reaction mixture. In one embodiment, water is added first to the reaction mixture of step (b), and then the neutralizing reagent is added as a solid or a solution to the reaction mixture.

[00168] In one embodiment, the quenched composition is stirred for about 15 minutes, about 30 minutes, about 45 minutes, about 1 hour, about 1.5 hour, about 2 hour, or more than about 2 hour. In one embodiment the quenched composition is stirred at the temperature of about 15˚C, about 10˚C, about 5˚C, or about 0˚C. In one embodiment, the quenched composition is stirred at the temperature of less than about 10˚C. In one embodiment, metallic oxide, such as, for example, stannic oxide or ferric oxide, settles to the bottom of the reaction vessel.

[00169] In one embodiment, the quenched composition of the reaction of step (b) is filtered. In one embodiment, the quenched composition of the reaction of step (b) is

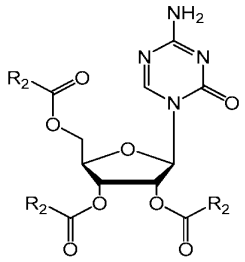
filtered through Hyflo Super Gel®. In one embodiment, the quenched composition of the reaction of step (b) is filtered through Celite®. In one embodiment, the filter cake is washed with an organic solvent, such as an organic non-polar solvent with low water solubility. In one embodiment, the quenching composition of the reaction of step (b) is filtered at a temperature of about 15˚C, about 10˚C, about 5˚C, or about 0˚C. In one embodiment, the quenching composition of the reaction of step (b) is filtered at a temperature of less than about 10˚C.

[00170] In one embodiment, the filtrate of the quenched composition of the reaction of step (b) contains an organic phase and an aqueous phase. In one embodiment, the organic phase of the filtrate is separated from its aqueous phase, and the desired product generally presents in the organic phase. In one embodiment, the organic phase is further washed with water. In one embodiment, the filtrate (*e.g.*, the organic phase of the filtrate) is washed with an aqueous EDTA (ethylenediaminetetraacetic acid) salt solution. In one embodiment, the filtrate is washed with an aqueous EDTA disodium salt solution, such as a 10% EDTA disodium salt solution. In one embodiment, the washing, extracting, and/or phase separation of the filtrate is carried out at a temperature of about 15˚C, about 10˚C, about 5˚C, or about 0˚C. In one embodiment, the washing, extracting, and/or phase separation of the filtrate is carried out at a temperature of less than about 10˚C. In one embodiment, the filtrate (*e.g.*, the organic phase of the filtrate) is dried over an anhydrous salt, such as, for example, anhydrous sodium sulfate or anhydrous magnesium sulfate. In one embodiment, the solvent of the filtrate is distilled off to afford the protected 5-azacytidine as a solid residue. In one embodiment, methanol is added to the solid residue of the protected 5-azacytidine to re-suspend the product, and the solvent of this mixture is then distilled off to form a solid residue of the protected 5-azacytidine. In certain embodiments, the solid residue of the protected 5-azacytidine is a crystalline solid. In one embodiment, the solvent of the filtrate used in extraction (*e.g.,* dichloromethane) is partially distilled off to afford a concentrated solution of protected 5-azacytidine, followed by the addition of an alcohol, such as methanol, to the mixture, and followed by vacuum distillation to substantially remove the solvent (*e.g.*, dichloromethane) and form a slurry of the protected 5-azacytidine in alcohol, such as methanol.

[00171] As described herein, the exposure of protected 5-azacytidine to water can be minimized by using a non-polar dry organic solvent for the coupling step. Alternatively, if a dry organic polar solvent is used in the coupling step, the solvent can be removed and replaced with a dry non-polar organic solvent prior to quenching. The quenching

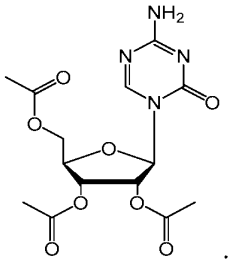
composition may be kept at a low temperature, such as less than about 10°C, to reduce the possibility of emulsion during aqueous extraction and the decomposition of the product during the work-up steps (*e.g.*, quenching, filtration, and extraction).

[00172] In one embodiment, the protected 5-azacytidine of step (b) is a compound of formula (C):



(C),

wherein R_1 and R_2 are defined herein elsewhere. In one embodiment, the protected 5-azacytidine is:



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[00173] In one embodiment, the protected 5-azacytidine is obtained following steps (a) and (b), in about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, or about 99% yield. In one embodiment, the yield is calculated based on 5-azacytosine used in step (a).

3. Step (c) — Deprotection

[00174] In one embodiment, the base used in step (c) is alkoxide, ammonia, or tetra-substituted ammonium hydroxide. In one embodiment, the base used in step (c) is alkoxide. In one embodiment, the base used in step (c) is ammonia. In one embodiment, the base used in step (c) is tetra-substituted ammonium hydroxide, such as for example, benzyl trimethyl ammonium hydroxide. In one embodiment, the base used in step (c) is sodium alkoxide. In one embodiment, the base used in step (c) is sodium methoxide.

[00175] In one embodiment, the alcohol used in step (c) is methanol. In one embodiment, the alcohol used in step (c) is ethanol. In one embodiment, the alcohol used in step (c) is isopropanol.

[00176] In one embodiment, the reaction of step (c) is carried out at room temperature. In one embodiment, the reaction of step (c) is carried out at a temperature of about 20°C. In one embodiment, the reaction of step (c) is carried out at a temperature of about 25°C. In one embodiment, the reaction of step (c) is carried out at a temperature of about 30°C.

[00177] The reaction time of the deprotection reaction of step (c) can vary from about 1 hr to about 24 hr, depending on the reaction temperature, the base used, and the concentration of the reagents in the reaction mixture. In general, the higher the reaction temperature, the shorter the reaction time, however, higher reaction temperature may give rise to side reactions or decomposition of the product. In specific embodiments, the reaction time of step (c) is about 1 hr, about 2 hr, about 3 hr, about 4 hr, about 5 hr, about 6 hr, about 7 hr, about 8 hr, about 9 hr, about 10 hr, about 12 hr, about 14 hr, about 16 hr, about 18 hr, about 20 hr, about 22 hr, or about 24 hr. In certain embodiments, the reaction time is about 6 hr, about 8 hr, about 10 hr, about 12 hr, about 14 hr, about 16 hr, about 18 hr, about 20 hr, about 22 hr, or about 24 hr at a reaction temperature of between about 25°C and about 30°C. In some embodiments, the progress of the reaction is monitored, such as by taking an aliquot of the reaction mixture and passing it through HPLC. In one embodiment, the reaction is quenched when the reaction is determined to be substantially complete, *e.g.*, via reaction progress monitoring.

[00178] In one embodiment, the reaction of step (c) is carried out under an inert atmosphere. In one embodiment, the reaction of step (c) is carried out under nitrogen. In one embodiment, the reaction of step (c) is carried out under argon.

[00179] In one embodiment, the reaction of step (c) is performed by stirring the protected 5-azacytidine in an alcohol, adding the base, and stirring the mixture at ambient temperature until the reaction is substantially complete. In one embodiment, the pH value of the reaction mixture is above about pH 10 after the base is added. In one embodiment, additional base is added until the pH value of the reaction mixture is above about pH 10.

[00180] In one embodiment, the 5-azacytidine of step (c) is collected by filtration. In one embodiment, the 5-azacytidine of step (c) is washed with a non-aqueous solvent, including but not limited to, an alcohol, such as methanol. In one embodiment, the 5-azacytidine of step (c) is dried under vacuum (*e.g.*, 10–15 mmHg). In one embodiment,

the 5-azacytidine of step (c) is dried at elevated temperature (*e.g.*, about 60˚C) under vacuum (*e.g.*, 10–15 mmHg).

[00181] In one embodiment, the 5-azacytidine is obtained following steps (a)–(c), in about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, or about 99% yield. In one embodiment, the yield is calculated based on 5-azacytosine used in step (a).

4. Step (d) — Salt Formation

[00182] In one embodiment, the acid used for salt formation in step (d) is an organic acid or an inorganic acid, including, but not limited to, acid capable of forming a pharmaceutically acceptable salt. In specific embodiments, the acid includes, but is not limited to, hydrochloric acid, hydrobromic acid, sulfuric acid, and methanesulfonic acid. In some embodiments, the acid used in step (d) is hydrochloric acid.

[00183] In one embodiment, the organic solvent used in step (d) is methanol, ethanol, isopropanol, ethyl acetate, tetrahydrofuran, acetone, methyl ethyl ketone, diethyl ether, methyl *t*-butyl ether, acetonitrile, *N*-methyl pyrrolidinone, dimethylformamide, dimethyl sulfoxide, dichloromethane, or chloroform, or the like, or mixtures thereof. In one embodiment, the organic solvent in step (d) is an alcohol or a mixture thereof. In some embodiments, the organic solvent in step (d) is methanol. In some embodiments, the organic solvent in step (d) is ethanol. In some embodiments, the organic solvent in step (d) is isopropanol.

[00184] In one embodiment, the salt formation reaction of step (d) is performed by stirring 5-azacytidine in an organic solvent, adding an acid, and stirring the mixture at ambient temperature.

[00185] In one embodiment, a molar excess of acid (relative to 5-azacytidine) is used. In one embodiment, the molar ratio between the acid and 5-azacytidine is about 1:1.

[00186] In one embodiment, the reaction of step (d) is carried out at room temperature. In one embodiment, the reaction of step (d) is carried out at a temperature of about 20˚C. In one embodiment, the reaction of step (d) is carried out at a temperature of about 25˚C. In one embodiment, the reaction of step (d) is carried out at a temperature of about 30˚C. In one embodiment, the reaction of step (d) is carried out at a temperature of less than about 25˚C. In one embodiment, the reaction of step (d) is carried out at a temperature of between about 25˚C and about 30˚C.

[00187] In one embodiment, the reaction of step (d) is carried out under an inert atmosphere. In one embodiment, the reaction of step (d) is carried out under nitrogen. In one embodiment, the reaction of step (d) is carried out under argon.

[00188] In one embodiment, the reaction time of the reaction of step (d) can vary from about 0.5 hr to about 24 hr. In one embodiment, the reaction time is about 0.5 hr, about 1 hr, about 2 hr, about 3 hr, about 4 hr, about 5 hr, about 6 hr, about 7 hr, about 8 hr, about 9 hr, about 10 hr, about 12 hr, about 14 hr, about 16 hr, about 18 hr, about 20 hr, about 22 hr, or about 24 hr. In one embodiment, the reaction time is about 1 hr. In one embodiment, the reaction time is about 2 hr. In one embodiment, the reaction time is about 3 hr. In one embodiment, the reaction time is about 4 hr. In some embodiments, the reaction mass first becomes a clear solution and then becomes a suspension, wherein the salt is crystallized from the reaction mass. In one embodiment, the reaction mixture of step (d) is cooled to a temperature of less than about 20˚C, less than about 15˚C, less than about 10˚C, or less than about 5˚C, to facilitate the crystallization of the salt.

[00189] In one embodiment, the salt of 5-azacytidine of step (d) is collected by filtration. In one embodiment, the salt of 5-azacytidine of step (d) is washed with an organic solvent, including but not limited to, an alcohol, such as methanol.

[00190] In one embodiment, the salt of 5-azacytidine of step (d) is isolated by concentrating the reaction mixture of step (d), followed by filtration. In one embodiment, the salt of 5-azacytidine of step (d) is isolated by concentrating the reaction mixture of step (d) under vacuum (*e.g.*, 10–15 mmHg) to remove volatile solvent.

[00191] In one embodiment, the salt of 5-azacytidine of step (d) is dried under vacuum (*e.g.*, 10–15 mmHg). In one embodiment, the salt of 5-azacytidine of step (d) is dried under vacuum (*e.g.*, 10–15 mmHg) at an elevated temperature (*e.g.*, about 50˚C, or about 60˚C).

[00192] In one embodiment, a salt of 5-azacytidine is obtained following step (d), in about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 99%, or about 99.9% yield. In one embodiment, the yield is calculated based on 5-azacytidine used in the salt formation reaction.

[00193] In one embodiment, the salt of 5-azacytidine obtained from step (d) is substantially pure. In one embodiment, the salt of 5-azacytidine obtained from step (d) is substantially physically pure. In one embodiment, the salt of 5-azacytidine obtained from step (d) is substantially chemically pure. In one embodiment, the salt of 5-azacytidine

obtained from step (d) is substantially free of impurities, such as, *e.g.*, a metal-based impurity. In one embodiment, the salt of 5-azacytidine from step (d) is obtained as a crystalline material that is substantially chemically and/or physically pure.

[00194] In one embodiment, the chemical purity of the salt of 5-azacytidine obtained from step (d) is greater than about 90% w/w, greater than about 95% w/w, greater than about 96% w/w, greater than about 97% w/w, greater than about 98% w/w, greater than about 99% w/w, greater than about 99.5% w/w, greater than about 99.8% w/w, greater than about 99.9% w/w, greater than about 99.95% w/w, greater than about 99.98% w/w, or greater than about 99.99% w/w relative to the total batch.

[00195] In one embodiment, the physical purity of the salt of 5-azacytidine obtained from step (d) is greater than about 90% w/w, greater than about 95% w/w, greater than about 96% w/w, greater than about 97% w/w, greater than about 98% w/w, greater than about 99% w/w, greater than about 99.5% w/w, greater than about 99.8% w/w, greater than about 99.9% w/w, greater than about 99.95% w/w, greater than about 99.98% w/w, or greater than about 99.99% w/w relative to the total batch.

[00196] In one embodiment, the total metal-based impurities in the salt of 5-azacytidine obtained from step (d) is less than about 500 ppm w/w, less than about 200 ppm w/w, less than about 100 ppm w/w, less than about 50 ppm w/w, less than about 20 ppm w/w, less than about 10 ppm w/w, less than about 5 ppm w/w, less than about 2 ppm w/w, less than about 1 ppm w/w, less than about 0.5 ppm w/w, less than about 0.2 ppm w/w, or less than about 0.1 ppm w/w relative to the total batch. In one embodiment, an individual metal based impurity, such as for example, tin or iron content, in the salt of 5-azacytidine obtained from step (d) is less than about 500 ppm w/w, less than about 200 ppm w/w, less than about 100 ppm w/w, less than about 50 ppm w/w, less than about 20 ppm w/w, less than about 10 ppm w/w, less than about 5 ppm w/w, less than about 2 ppm w/w, less than about 1 ppm w/w, less than about 0.5 ppm w/w, less than about 0.2 ppm w/w, less than about 0.1 ppm w/w, less than about 0.05 ppm w/w, less than about 0.02 ppm w/w, or less than about 0.01 ppm w/w relative to the total batch.

5. Step (e) — Free Base Formation

[00197] In one embodiment, the base used to form the 5-azacytidine free base in step (e) is an organic base or an inorganic base. In one embodiment, the base used in step (e) is an organic base, including but not limited to, triethylamine, diisopropylethyl amine,

pyridine, diisopropylamine, 2,6-lutidine, *N*-methylmorpholine, *N,N*-dicyclohexylmethyl amine, and the like. In some embodiments, the base in step (e) is triethylamine.

[00198] In one embodiment, the organic solvent used in step (e) is methanol, ethanol, isopropanol, ethyl acetate, tetrahydrofuran, acetone, methyl ethyl ketone, diethyl ether, methyl *t*-butyl ether, acetonitrile, *N*-methyl pyrrolidinone, dimethylformamide, dimethyl sulfoxide, dichloromethane, or chloroform, or the like, or mixtures thereof. In one embodiment, the organic solvent in step (e) is alcohol or a mixture thereof. In some embodiments, the organic solvent in step (e) is methanol. In some embodiments, the organic solvent in step (e) is ethanol. In some embodiments, the organic solvent in step (e) is isopropanol.

[00199] In one embodiment, the free base formation reaction of step (e) is performed by stirring a salt of 5-azacytidine in an organic solvent, adding a base, and stirring the mixture at ambient temperature.

[00200] In one embodiment, a molar excess of base (relative to 5-azacytidine) is used. In one embodiment, the molar ratio between the base and 5-azacytidine is about 1:1.

[00201] In one embodiment, the reaction of step (e) is carried out at room temperature. In one embodiment, the reaction of step (e) is carried out at a temperature of about 20°C. In one embodiment, the reaction of step (e) is carried out at a temperature of about 25°C. In one embodiment, the reaction of step (e) is carried out at a temperature of about 30°C. In one embodiment, the reaction of step (e) is carried out at a temperature of less than about 30°C. In one embodiment, the reaction of step (e) is carried out at a temperature of between about 25°C and about 30°C.

[00202] In one embodiment, the reaction of step (e) is carried out under an inert atmosphere. In one embodiment, the reaction of step (e) is carried out under nitrogen. In one embodiment, the reaction of step (e) is carried out under argon.

[00203] In one embodiment, the reaction time of the reaction of step (e) can vary from about 0.5 hr to about 24 hr. In one embodiment, the reaction time is about 0.5 hr, about 1 hr, about 2 hr, about 3 hr, about 4 hr, about 5 hr, about 6 hr, about 7 hr, about 8 hr, about 9 hr, about 10 hr, about 12 hr, about 14 hr, about 16 hr, about 18 hr, about 20 hr, about 22 hr, or about 24 hr. In one embodiment, the reaction time is about 1 hr. In one embodiment, the reaction time is about 2 hr. In one embodiment, the reaction time is about 3 hr. In one embodiment, the reaction time is about 4 hr. In some embodiments, the reaction mass is stirred as a slurry upon formation of the free base of 5-azacytidine. In one embodiment, the reaction mixture of step (e) is cooled to a temperature of less than about

20˚C, less than about 15˚C, less than about 10˚C, or less than about 5˚C, before it is filtered.

[00204] In one embodiment, the free base of 5-azacytidine of step (e) is collected by filtration. In one embodiment, the free base of 5-azacytidine of step (e) is washed with an organic solvent, such as for example, an alcohol, such as methanol. In one embodiment, the washing is continued until the free base of 5-azacytidine of step (e) is substantially free of impurities, such as for example, the acid addition salt counter ion (such as, *e.g.*, chloride). In one embodiment, the free base of 5-azacytidine of step (e) is substantially free of impurities, including but not limited to, metal-based impurity and acid addition salt counter ion.

[00205] In one embodiment, the free base of 5-azacytidine of step (e) is dried under vacuum (*e.g.* 10–15 mmHg). In one embodiment, the free base of 5-azacytidine of step (e) is dried under vacuum (*e.g.* 10–15 mmHg) at an elevated temperature (*e.g.* about 50˚C, or about 60˚C).

[00206] In one embodiment, a free base of 5-azacytidine is obtained following step (e), in about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 99%, or about 99.9% yield. In one embodiment, the yield is calculated based on the salt of 5-azacytidine used.

[00207] In one embodiment, the free base of 5-azacytidine obtained from step (e) is substantially pure. In one embodiment, the free base of 5-azacytidine obtained from step (e) is substantially physically pure. In one embodiment, the free base of 5-azacytidine obtained from step (e) is substantially chemically pure. In one embodiment, the free base of 5-azacytidine obtained from step (e) is substantially free of impurities, such as, *e.g.*, a metal-based impurity and/or acid addition salt counter ion. In one embodiment, the free base of 5-azacytidine from step (e) is obtained as a crystalline material that is substantially chemically and/or physically pure.

[00208] In one embodiment, the chemical purity of the free base of 5-azacytidine obtained from step (e) is greater than about 90% w/w, greater than about 95% w/w, greater than about 96% w/w, greater than about 97% w/w, greater than about 98% w/w, greater than about 99% w/w, greater than about 99.5% w/w, greater than about 99.8% w/w, greater than about 99.9% w/w, greater than about 99.95% w/w, greater than about 99.98% w/w, or greater than about 99.99% w/w relative to the total batch.

[00209] In one embodiment, the physical purity of the free base of 5-azacytidine obtained from step (e) is greater than about 90% w/w, greater than about 95% w/w, greater than about 96% w/w, greater than about 97% w/w, greater than about 98% w/w, greater than about 99% w/w, greater than about 99.5% w/w, greater than about 99.8% w/w, greater than about 99.9% w/w, greater than about 99.95% w/w, greater than about 99.98% w/w, or greater than about 99.99% w/w relative to the total batch.

[00210] In one embodiment, the total metal-based impurities in the free base of 5-azacytidine obtained from step (e) is less than about 500 ppm w/w, less than about 200 ppm w/w, less than about 100 ppm w/w, less than about 50 ppm w/w, less than about 20 ppm w/w, less than about 10 ppm w/w, less than about 5 ppm w/w, less than about 2 ppm w/w, less than about 1 ppm w/w, less than about 0.5 ppm w/w, less than about 0.2 ppm w/w, or less than about 0.1 ppm w/w relative to the total batch. In one embodiment, an individual metal based impurity, such as for example, tin or iron content, in the free base of 5-azacytidine obtained from step (e) is less than about 500 ppm w/w, less than about 200 ppm w/w, less than about 100 ppm w/w, less than about 50 ppm w/w, less than about 20 ppm w/w, less than about 10 ppm w/w, less than about 5 ppm w/w, less than about 2 ppm w/w, less than about 1 ppm w/w, less than about 0.5 ppm w/w, less than about 0.2 ppm w/w, less than about 0.1 ppm w/w, less than about 0.05 ppm w/w, less than about 0.02 ppm w/w, or less than about 0.01 ppm w/w relative to the total batch.

6. Step (f) — Re-crystallization

[00211] In one embodiment, step (f) comprises the steps of:

(1) dissolving 5-azacytidine free base from step (e) in dimethylsulfoxide at a temperature sufficient to allow the 5-azacytidine to dissolve; and optionally filtering the solution to remove insoluble particles;

(2) adding an anti-solvent to the solution of step (1); and

(3) cooling the mixture of step (2) wherein 5-azacytidine re-crystallizes.

[00212] In one embodiment, 5-azacytidine is dissolved in dimethylsulfoxide (DMSO) in step (f)(1) at an elevated temperature, such as for example, at a temperature of about 40˚C, about 45˚C, about 50˚C, about 55˚C, about 60˚C, about 65˚C, about 70˚C, about 75˚C, about 80˚C, about 85˚C, about 90˚C, about 95˚C, or about 100˚C. In one embodiment, the optional filtration in step (f)(1) is carried out at an elevated temperature, such as for example, at a temperature of about 40˚C, about 45˚C, about 50˚C, about 55˚C, about 60˚C, about 65˚C, about 70˚C, about 75˚C, about 80˚C, about 85˚C, about 90˚C,

about 95˚C, or about 100˚C. In one embodiment, optionally, the insoluble particles are washed with hot DMSO.

[00213] In one embodiment, the anti-solvent of step (f)(2) is an alcohol. In one embodiment, the anti-solvent of step (f)(2) is methanol. In one embodiment, the anti-solvent is added slowly to the 5-azacytidine DMSO solution, at a temperature of about 40˚C, about 45˚C, about 50˚C, about 55˚C, about 60˚C, about 65˚C, about 70˚C, about 75˚C, about 80˚C, or about 85˚C. In one embodiment, the anti-solvent is methanol, which is added slowed to the 5-azacytidine DMSO solution at a temperature of between about 70˚C and about 80˚C, between about 60˚C and about 70˚C, between about 50˚C and about 60˚C, between about 40˚C and about 50˚C, or between about 30˚C and about 40˚C.

[00214] In one embodiment, the mixture of step (f)(3) is cooled to a temperature of about 35˚C, about 30˚C, about 25˚C, about 20˚C, about 15˚C, about 10˚C, about 5˚C or about 0˚C. In one embodiment, the mixture of step (f)(3) is cooled to a temperature of between about 25˚C and about 30˚C. In one embodiment, the mixture of step (f)(3) is cooled slowly over a period of about 1 hr, about 2 hr, about 3 hr, about 4 hr, about 5 hr, about 6 hr, about 7 hr, about 8 hr, about 9 hr, about 10 hr, about 11 hr, or about 12 hr. In one embodiment, after cooling, the mixture of step (f)(3) is stirred for a period of about 1 hr, about 2 hr, about 3 hr, about 4 hr, about 5 hr, about 6 hr, about 7 hr, about 8 hr, about 9 hr, about 10 hr, about 11 hr, about 12 hr, about 13 hr, about 14 hr, about 15 hr, about 16 hr, about 17 hr, about 18 hr, about 19 hr, about 20 hr, about 21 hr, about 22 hr, about 23 hr, or about 24 hr. In one embodiment, after cooling the mixture to a temperature of between about 25˚C and about 30˚C, the mixture is stirred for about 15 hr at a temperature of between about 25˚C and about 30˚C.

[00215] In specific embodiments, precipitation of the 5-azacytidine is induced upon anti-solvent addition. In specific embodiments, precipitation is induced upon cooling. In specific embodiments, precipitation is induced by both anti-solvent addition and cooling.

[00216] In one embodiment, step (f)(1) is carried out under an inert atmosphere. In one embodiment, step (f)(2) is carried out under an inert atmosphere. In one embodiment, step (f)(3) is carried out under an inert atmosphere.

[00217] In one embodiment, step (f) further comprises the steps of:

(4) collecting the re-crystallized 5-azacytidine from step (3) by filtration; and

(5) drying the 5-azacytidine from step (4) under vacuum.

[00218] In one embodiment, the filtration of step (f)(4) is carried under an inert atmosphere. In one embodiment, the solid product is washed with a solvent, such as,

methanol. In one embodiment, the solid product is washed with a solvent, which is the same solvent used at the anti-solvent in step (f)(2).

[00219] In one embodiment, the solid product is dried under vacuum (*e.g.* 10–15 mmHg). In one embodiment, the drying is carried out at room temperature. In one embodiment, the drying the carried out at an elevated temperature, for example, about 40˚C, about 45˚C, about 50˚C, about 55˚C, about 60˚C, about 65˚C, about 70˚C, about 75˚C, about 80˚C, about 85˚C, about 90˚C, about 95˚C, or about 100˚C. In one embodiment, the drying the carried out at a temperature of between about 85˚C and about 90˚C. In one embodiment, the drying is continued until the weight loss on drying falls below about 0.4% w/w. In one embodiment, the drying is carried out over a period of about 1 hr, about 2 hr, about 3 hr, about 4 hr, about 5 hr, about 6 hr, about 8 hr, about 10 hr, about 12 hr, about 14 hr, about 16 hr, about 18 hr, about 20 hr, about 22 hr, about 24 hr, about 36 hr, about 48 hr, about 60 hr, or about 72 hr.

[00220] In one embodiment, the re-crystallized 5-azacytidine is obtained following step (f), in about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 99%, or about 99.9% yield. In one embodiment, the yield is calculated based on the 5-azacytidine used for re-crystallization.

7. Additional Embodiments

[00221] In one embodiment, 5-azacytosine is first reacted with HMDS in the presence of ammonium sulfate to afford a silylated 5-azacytosine, which is coupled with 1,2,3,5-tetra-O-acetyl-β-D-ribofuranose in the presence of stannic chloride ($SnCl_4$) to afford the tri-acetyl protected 5-azacytidine. In some embodiments, the chemical and/or physical purity of the tri-acetyl protected coupling product is greater than about 50% w/w, greater than about 60% w/w, greater than about 70% w/w, greater than about 80% w/w, greater than about 90% w/w, or greater than about 95% w/w. In one embodiment, the chemical and/or physical purity of the tri-acetyl protected coupling product is greater than about 75% w/w. In some embodiments, the yield of the coupling reaction is between about 50% and about 99%, between about 60% and about 90%, between about 60% and about 80%, or between about 65% and about 75% (yield is based on chemical purity of the crude product). In one embodiment, the yield of the coupling reaction is about 70% (yield is based on chemical purity of the crude product).

[00222] In another embodiment, 5-azacytosine is first reacted with HMDS in the presence of ammonium sulfate to afford a silylated 5-azacytosine, which is coupled with 1,2,3,5-tetra-O-acetyl-β-D-ribofuranose in the presence of ferric chloride ($FeCl_3$) to afford the tri-acetyl protected 5-azacytidine. In some embodiments, the chemical and/or physical purity of the tri-acetyl protected coupling product is greater than about 40% w/w, greater than about 50% w/w, greater than about 60% w/w, greater than about 70% w/w, greater than about 80% w/w, or greater than about 90% w/w. In one embodiment, the chemical and/or physical purity of the tri-acetyl protected coupling product is greater than about 60% w/w. In some embodiments, the yield of the coupling reaction is between about 30% and about 99%, between about 40% and about 90%, between about 40% and about 80%, between about 40% and about 70%, between about 40% and about 60%, or between about 45% and about 55% (yield is based on chemical purity of the crude product). In one embodiment, the yield of the coupling reaction is about 50% (yield is based on chemical purity of the crude product).

[00223] In one embodiment, the acetyl protected 5-azacytidine is deacetylated with sodium methoxide solution in methanol to afford a crude product of 5-azacytidine. In some embodiments, the chemical and/or physical purity of the crude 5-azacytidine is greater than about 80% w/w, greater than about 90% w/w, greater than about 95% w/w, or greater than about 99% w/w, when $SnCl_4$ is used as the Lewis acid in the preceding coupling step. In one embodiment, the chemical and/or physical purity of the crude 5-azacytidine is greater than about 95% w/w, when $SnCl_4$ is used as the Lewis acid in the preceding coupling step. In other embodiments, the chemical and/or physical purity of the crude 5-azacytidine is greater than about 80% w/w, greater than about 90% w/w, greater than about 95% w/w, or greater than about 99% w/w, when $FeCl_3$ is used as the Lewis acid in the preceding coupling step. In one embodiment, the chemical and/or physical purity of the crude 5-azacytidine is greater than about 90% w/w, when $FeCl_3$ is used as the Lewis acid in the preceding coupling step. In some embodiments, the combined yield of the coupling step and the deprotection step is between about 40% and about 90%, between about 50% and about 75%, or between about 55% and about 65%, when $SnCl_4$ is used as the Lewis acid (yield is based on 5-azacytosine). In one embodiment, the combined yield of the coupling step and the deprotection step is about 60%, when $SnCl_4$ is used as the Lewis acid (yield is based on 5-azacytosine). In other embodiments, the combined yield of the coupling step and the deprotection step is between about 20% and about 80%,

between about 30% and about 65%, or between about 35% and about 45%, when $FeCl_3$ is used as the Lewis acid (yield is based on 5-azacytosine). In one embodiment, the combined yield of the coupling step and the deprotection step is about 40%, when $FeCl_3$ is used as the Lewis acid (yield is based on 5-azacytosine).

[00224] In one embodiment, the crude 5-azacytidine is treated with isopropanol-HCl (IPA-HCl) in methanol to form 5-azacytidine HCl salt. In some embodiments, the chemical and/or physical purity of the 5-azacytidine HCl salt is greater than about 90% w/w, greater than about 92% w/w, greater than about 94% w/w, greater than about 96% w/w, greater than about 98% w/w, greater than about 99% w/w, or greater than about 99.5% w/w. In one embodiment, the chemical and/or physical purity of the 5-azacytidine HCl salt is greater than about 98% w/w. In some embodiments, the yield of the salt formation step is about 60%, about 70%, about 80%, about 90%, about 95%, or about 99%, when $SnCl_4$ is used as the Lewis acid in the preceding coupling step. In one embodiment, the yield of the salt formation step is about 80%, when $SnCl_4$ is used as the Lewis acid in the preceding coupling step. In other embodiments, the yield of the salt formation step is about 50%, about 60%, about 70%, about 80%, about 90%, or about 95%, when $FeCl_3$ is used as the Lewis acid in the preceding coupling step. In one embodiment, the yield of the salt formation step is about 72%, when $FeCl_3$ is used as the Lewis acid in the preceding coupling step.

[00225] In one embodiment, the 5-azacytidine HCl salt is treated with triethylamine in the presence of methanol to afford the free base of 5-azacytidine. In some embodiments, the chemical and/or physical purity of the 5-azacytidine free base is greater than about 90% w/w, greater than about 95% w/w, greater than about 98% w/w, greater than about 99% w/w, greater than about 99.5% w/w, or greater than about 99.9% w/w. In one embodiment, the chemical and/or physical purity of the 5-azacytidine free base is greater than about 99% w/w. In some embodiments, the yield of the free base formation step is about 70%, about 80%, about 90%, about 95%, about 98%, or about 99%, when $SnCl_4$ is used as the Lewis acid in the preceding coupling step. In one embodiment, the yield of the free base formation step is about 95%, when $SnCl_4$ is used as the Lewis acid in the preceding coupling step. In other embodiments, the yield of the salt formation step is about 70%, about 80%, about 90%, about 95%, about 98%, or about 99%, when $FeCl_3$ is used as the Lewis acid in the preceding coupling step. In one embodiment, the yield of the

salt formation step is about 99%, when $FeCl_3$ is used as the Lewis acid in the preceding coupling step.

[00226] In one embodiment, the 5-azacytidine free base is re-crystallized in DMSO and methanol to afford 5-azacytidine as the final product. In some embodiments, the chemical and/or physical purity of the 5-azacytidine final product is greater than about 90% w/w, greater than about 95% w/w, greater than about 98% w/w, greater than about 99% w/w, greater than about 99.5% w/w, greater than about 99.8% w/w, or greater than about 99.9% w/w. In one embodiment, the chemical and/or physical purity of the 5-azacytidine final product is greater than about 99% w/w. In some embodiments, the yield of the re-crystallization step is about 70%, about 80%, about 85%, about 90%, about 95%, or about 99%, when $SnCl_4$ is used as the Lewis acid in the preceding coupling step. In one embodiment, the yield of the re-crystallization step is about 94%, when $SnCl_4$ is used as the Lewis acid in the preceding coupling step. In other embodiments, the yield of the re-crystallization step is about 70%, about 80%, about 85%, about 90%, about 95%, or about 99%, when $FeCl_3$ is used as the Lewis acid in the preceding coupling step. In one embodiment, the yield of the re-crystallization step is about 85%, when $FeCl_3$ is used as the Lewis acid in the preceding coupling step.

[00227] In one embodiment, provided herein is a process of preparing isotopically labeled 5-azacytidine, wherein the process uses one or more isotopically labeled starting material. In certain embodiments, a deuterium labeled starting material is used. In certain embodiments, a tritium labeled starting material is used. In certain embodiments, a ^{13}C labeled starting material is used. In certain embodiments, a ^{15}N labeled starting material is used. In certain embodiments, a ^{17}O labeled starting material is used. In certain embodiments, the isotopically labeled starting material has two or more isotopic labels, including, but not limited to, deuterium, tritium, ^{13}C, ^{15}N, and/or ^{17}O labels. In certain embodiments, provided herein is a process to prepare 5-azacytidine with one or more isotopic label(s), including, but not limited to, deuterium, tritium, ^{13}C, ^{15}N, and/or ^{17}O labels. In one embodiment, the isotopically labeled 5-azacytidine is substantially chemically and/or physically pure. In certain embodiments, at least one isotopically labeled starting material is used in step (a). In certain embodiments, at least one isotopically labeled starting material is used in step (b).

C. Salts of 5-Azacytidine

[00228] In one embodiment, provided herein are acid addition salts of 5-azacytidine, wherein the acid used in the preparation of the 5-azacytidine salt is an organic acid or an inorganic acid, including, but not limited to, hydrochloric acid, hydrobromic acid, sulfuric acid, and methanesulfonic acid. In one embodiment, provided herein is a salt of 5-azacytidine, including, but not limited to hydrochloride salt, hydrobromide salt, sulfate salt (including, *e.g.,* bisulfate salt and hemisulfate salt), and methanesulfonate salt (*i.e.*, mesylate salt). In one embodiment, provided herein is 5-azacytidine hemisulfate salt. In one embodiment, provided herein is 5-azacytidine mono-hydrochloride salt. In one embodiment, provided herein is 5-azacytidine mono-hydrobromide salt. In one embodiment, provided herein is 5-azacytidine hemisulfate salt, which is solvated with methanol. In one embodiment, the 5-azacytidine hemisulfate salt is solvated with methanol and the molar ratio of 5-azacytidine and methanol is about 1:1. In one embodiment, provided herein is 5-azacytidine mesylate salt, which is solvated with methanol. In one embodiment, the 5-azacytidine mesylate salt is solvated with methanol and the molar ratio of 5-azacytidine and methanol is about 1:1. In one embodiment, provided herein is a salt or solvate of 5-azacytidine that is substantially chemically and/or physically pure. In one embodiment, provided herein is a salt or solvate of 5-azacytidine that is substantially free of one or more impurities, such as for example, a metal-based impurity.

[00229] In one embodiment, provided herein is a pharmaceutically acceptable salt of 5-azacytidine, including, but not limited to hydrochloric acid salt, hydrobromic acid salt, sulfuric acid salt, and methanesulfonic acid salt. In one embodiment, provided herein is a pharmaceutically acceptable salt of 5-azacytidine that is substantially chemically and/or physically pure. In one embodiment, provided herein is a pharmaceutically acceptable salt of 5-azacytidine that is substantially free of one or more impurities.

[00230] In one embodiment, provided herein is a mono-hydrochloride salt of 5-azacytidine that is substantially chemically and/or physically pure. In one embodiment, provided herein is a 5-azacytidine mono-hydrochloride salt that is substantially free of one or more impurities. In specific embodiments, the 5-azacytidine mono-hydrochloride salt is substantially pure. In one embodiment, the 5-azacytidine mono-hydrochloride salt is substantially physically pure. In one embodiment, the 5-azacytidine mono-hydrochloride salt is substantially chemically pure.

[00231] In one embodiment, the chemical and/or physical purity of the 5-azacytidine mono-hydrochloride salt is greater than about 95% w/w, greater than about 96% w/w,

greater than about 97% w/w, greater than about 98% w/w, greater than about 99% w/w, greater than about 99.5% w/w, greater than about 99.8% w/w, greater than about 99.9% w/w, greater than about 99.95% w/w, greater than about 99.98% w/w, or greater than about 99.99% w/w. In one embodiment, the total impurities in the 5-azacytidine mono-hydrochloride salt is less than about 5% w/w, less than about 4% w/w, less than about 3% w/w, less than about 2% w/w, less than about 1% w/w, less than about 0.5% w/w, less than about 0.2% w/w, less than about 0.1% w/w, less than about 0.05% w/w, less than about 0.02% w/w, less than about 0.01% w/w, less than about 0.005% w/w, or less than about 0.001% w/w. In one embodiment, an individual impurity component in the 5-azacytidine mono-hydrochloride salt is less than about 5% w/w, less than about 2% w/w, less than about 1% w/w, less than about 0.9% w/w, less than about 0.8% w/w, less than about 0.7% w/w, less than about 0.6% w/w, less than about 0.5% w/w, less than about 0.4% w/w, less than about 0.3% w/w, less than about 0.2% w/w, less than about 0.1% w/w, less than about 0.05% w/w, less than about 0.01% w/w, less than about 0.005% w/w, less than about 0.001% w/w, less than about 0.0005% w/w, or less than about 0.0001% w/w. In one embodiment, the total metal-based impurities in the 5-azacytidine mono-hydrochloride salt is less than about 500 ppm w/w, less than about 200 ppm w/w, less than about 100 ppm w/w, less than about 50 ppm w/w, less than about 20 ppm w/w, less than about 10 ppm w/w, less than about 5 ppm w/w, less than about 2 ppm w/w, less than about 1 ppm w/w, less than about 0.5 ppm w/w, less than about 0.2 ppm w/w, or less than about 0.1 ppm w/w. In one embodiment, an individual metal based impurity, such as for example, tin or iron content, in the 5-azacytidine mono-hydrochloride salt is less than about 500 ppm w/w, less than about 200 ppm w/w, less than about 100 ppm w/w, less than about 50 ppm w/w, less than about 20 ppm w/w, less than about 10 ppm w/w, less than about 5 ppm w/w, less than about 2 ppm w/w, less than about 1 ppm w/w, less than about 0.5 ppm w/w, less than about 0.2 ppm w/w, less than about 0.1 ppm w/w, less than about 0.05 ppm w/w, less than about 0.02 ppm w/w, or less than about 0.01 ppm w/w.

[00232] In one embodiment, provided herein is a free base of 5-azacytidine having any three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, or fifteen of the peaks as listed in **Table 18** or **Table 20** below.

[00233] In one embodiment, provided herein is a mono-hydrochloride salt of 5-azacytidine having any three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, or fifteen of the peaks as listed in **Table 24** or **Table 26** below.

[00234] In one embodiment, provided herein is a sulfate salt of 5-azacytidine (*e.g.*, hemisulfate salt) having any three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, or fifteen of the peaks as listed in **Table 28** below.

[00235] In one embodiment, provided herein is a mesylate salt of 5-azacytidine having any three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, or fifteen of the peaks as listed in **Table 30** below.

[00236] In one embodiment, provided herein is a hydrobromide salt of 5-azacytidine having any three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, or fifteen of the peaks as listed in **Table 32** below.

[00237] Also provided herein is a method for preparing an acid addition salt of 5-azacytidine, comprising mixing 5-azacytidine with an acid in an organic solvent, and isolating the salt of 5-azacytidine.

[00238] In one embodiment, the 5-azacytidine free base used for salt formation is prepared using the processes described herein. In other embodiments, the 5-azacytidine used for salt formation is obtained from a commercial source. In other embodiments, the 5-azacytidine used for salt formation is prepared following a literature procedure.

[00239] In one embodiment, 5-azacytidine is mixed with an acid in a molar ratio of about 1:1. In other embodiments, excess acid is used to form the 5-azacytidine salt.

[00240] In one embodiment, the acid used for salt formation is an organic acid or an inorganic acid, wherein examples of organic acids and inorganic acids are provided herein elsewhere. Non-limiting examples of suitable acids include methanesulfonic acid, trifluoroacetic acid, *p*-toluenesulfonic acid, hydrochoric acid, hydrobromic acid, nitric acid, sulfuric acid, and phosphoric acid.

[00241] In one embodiment, the organic solvent used in making a salt of 5-azacytidine is methanol, ethanol, isopropanol, ethyl acetate, tetrahydrofuran, acetone, methyl ethyl ketone, diethyl ether, methyl *t*-butyl ether, acetonitrile, *N*-methyl pyrrolidinone, dimethyl sulfoxide, dimethylformamide, dichloromethane, or chloroform, or a combination of one or more thereof. In one embodiment, the organic solvent used for salt formation is an alcohol, such as for example, methanol, ethanol, and isopropanol, or a mixture of one or more thereof. In one embodiment, the organic solvent used for salt formation is methanol.

[00242] In one embodiment, the salt of 5-azacytidine is formed by stirring 5-azacytidine in an organic solvent system, adding an acid, and stirring the mixture at ambient temperature. In one embodiment, the salt formation reaction is carried out under an inert atmosphere. In one embodiment, the salt of 5-azacytidine is collected by

filtration. In one embodiment, the salt of 5-azacytidine is washed with an organic solvent, such as methanol. In one embodiment, the salt of 5-azacytidine is isolated by concentrating the mixture containing the 5-azacytidine salt (obtained as described above) under vacuum (*e.g.* 10–15 mmHg) to remove volatile solvent, and isolating the salt by filtration.

[00243] In one embodiment, the salt of 5-azacytidine is dried under vacuum (*e.g.* 10–15 mmHg). In one embodiment, the salt of 5-azacytidine of step (d) is dried under vacuum (*e.g.* 10–15 mmHg) at an elevated temperature (*e.g.* about 50˚C, or about 60˚C).

VI. EXAMPLES

[00244] Certain embodiments are illustrated by the following non-limiting examples.

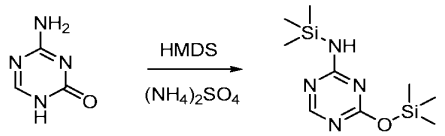
[00245] In the examples below, unless otherwise indicated, all temperatures are set forth in degrees Celsius and all parts and percentages are by weight. Reagents may be purchased from commercial suppliers, such as, *e.g.,* Sigma–Aldrich® Chemical Co., and may be used without further purification unless otherwise indicated. Reagents may also be prepared following standard literature procedures known to those skilled in the art. Solvents may be purchased, for example, from Sigma–Aldrich®, and may be used as received or may be purified using standard methods known to those skilled in the art, unless otherwise indicated.

[00246] Unless otherwise specified, the reactions set forth below were done generally at ambient temperature. Reactions were assayed by HPLC, and terminated as judged by the consumption of starting material.

[00247] The compound structures and purities in the examples below were confirmed by one or more of the following methods: proton nuclear magnetic resonance (^{1}H NMR) spectroscopy, ^{13}C NMR spectroscopy, mass spectroscopy, infrared spectroscopy, melting point, X-ray crystallography, and/or HPLC. ^{1}H NMR spectra were determined using a NMR spectrometer operating at a certain field strength. Chemical shifts are reported in parts per million (ppm, δ) downfield from an internal standard, such as TMS. Alternatively, ^{1}H NMR spectra were referenced to signals from residual protons in deuterated solvents as follows: $CDCl_3$ = 7.25 ppm; DMSO-d_6 = 2.49 ppm; C_6D_6 = 7.16 ppm; CD_3OD = 3.30 ppm. Peak multiplicities are designated as follows: s, singlet; d, doublet; dd, doublet of doublets; t, triplet; dt, doublet of triplets; q, quartet; br, broadened; and m, multiplet. Coupling constants are given in Hertz (Hz). Mass spectra (MS) data were obtained using a mass spectrometer with APCI or ESI ionization.

A. The Stannic Chloride Route

1. Preparation of Silylated 5-Azacytosine

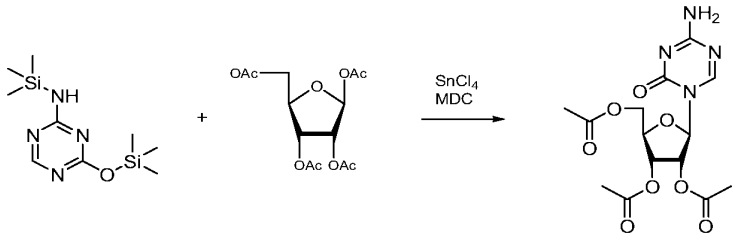


[00248] 5-Azacytosine (100.0 g, 0.8921 mol, purity ≥ 98%) and hexamethyldisilazane (HMDS) (700.0 mL, 541.8 g, 3.3568 mol, purity ≥ 98%) were charged into a 3-L 4-neck round bottom flask at 25–30˚C under nitrogen atmosphere. Ammonium sulfate (5.0 g, 0.0378 mol) was added. The mixture was gradually heated to reflux at 125–130˚C. The reflux was maintained for 6 hours. Typically, the reaction mass became a clear solution after 2–4 hours (*e.g.,* about 3 hours), and the reaction was complete as soon as a clear solution was formed.

[00249] The reaction mass was gradually cooled to 40–50˚C. HMDS was distilled off at 40–50˚C under vacuum (10–15 mmHg) to give a white solid. Nitrogen was used to break the vacuum over the solid. Toluene (200.0 mL) was added to the residue at 25–30˚C, and the solvent was distilled off at 40–50 ˚C under vacuum (10–15 mmHg) to yield a solid. Nitrogen was used to break the vacuum over the solid. The solid was gradually cooled to 25–30˚C, and carried through to the next step. HMDS was recovered in 75–80 % yield with a purity of 90–95%.

[00250] The silylation reaction was also performed using 4, 5, 7, 10, and 14 volumes of HMDS (e.g., 400, 500, 700, 1000, or 1400 mL HMDS to 100 g 5-azacytosine).

2. Preparation of Acetyl-Protected 5-Azacytidine (Coupling)



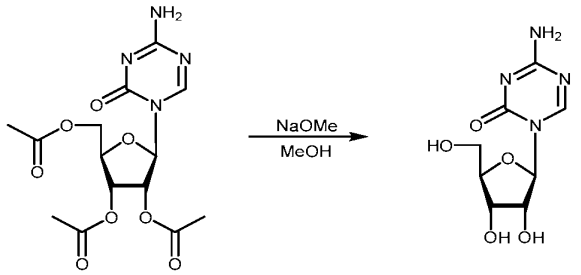
[00251] To silylated 5-azacytosine (Example A-1) was added dichloromethane (1.5 L). The mixture was stirred for 10 minutes under nitrogen atmosphere at 25–30˚C to obtain a clear solution. The solution was gradually cooled to 0–5˚C. 1,2,3,5-Tetra-O-acetyl-β-D-ribofuranose (255.5 g, 0.8029 mol) was added in one lot at 0–5˚C under nitrogen atmosphere. The mixture was stirred for 10 minutes at 0–5˚C to obtain a clear solution.

Stannic chloride (255.6 g, 0.9813 mol) was added drop-wise (addition was slightly exothermic) at ≤ 10˚C over a period of 1 hour. The reaction mass was stirred for 5 hours at 0–5˚C under nitrogen atmosphere.

[00252] The progress of the reaction was checked by HPLC. 5 g of reaction mass was withdrawn and neutralized with saturated aqueous $NaHCO_3$ solution at 10˚C. The dichloromethane layer was separated and submitted for IPC-HPLC (In-Process-Control-HPLC). Once IPC had been met (5-azacytosine no more than 0.5% by HPLC), the reaction mass was transferred to a 5-L round bottom flask for work up.

[00253] To the reaction mass was added dichloromethane (1.0 L) and sodium bicarbonate (800.0 g) at ≤ 10˚C. Chilled water (1.0 L) was added drop-wise (exothermic) at ≤ 10˚C over 30 minutes. The mixture was stirred for 30 minutes at ≤ 10˚C. After 15–30 minutes, a white solid (tin oxide) settled at the bottom of the flask. The mixture was filtered through Hyflo®, and washed with dichloromethane (0.5 L). The organic layer was separated at ≤ 10˚C, and washed with water (0.75 L) at ≤ 10˚C. The organic layer was washed with 10% EDTA disodium salt solution twice (150.0 g salt, 2 x 750 mL) and water once (1.0 L) at ≤ 10˚C. The organic layer was dried over anhydrous sodium sulfate, and the solvent was distilled off at 40–45˚C under atmospheric conditions, and further dried at 40–45˚C under vacuum (10–15 mmHg) to give a sticky foaming solid. To the residue was added methanol (200.0 mL) at 30–35˚C. The solvent was then distilled off at 40–45˚C under vacuum (10–15 mmHg), and degassed under vacuum for 30 minutes to afford 2',3',5'-triacetyl-5-azacytidine as a solid (315.0 g, white to off-white crystalline solid). The average output of 2',3',5'-triacetyl-5-azacytidine over multiple runs was about 305.2 g, with an average purity of about 83.7%, and an average yield of about 77.2% (% yield takes into account the HPLC purity of the product). Over five runs, the maximum yield of 2',3',5'-triacetyl-5-azacytidine was about 81.5% (% yield takes into account the HPLC purity of the product). Over five runs, the maximum HPLC purity of the product was about 87.8%.

3. Preparation of 5-Azacytidine (Deprotection)



[00254] To 2',3',5'-triacetyl-5-azacytidine (315.0 g, Example A-2) was added methanol (2.0 L) at 25–30˚C. The slurry was stirred for 10 minutes at 25–30˚C, and 25% sodium methoxide in methanol (40.0 mL) was added slowly at 25–30˚C under nitrogen atmosphere. After addition, the reaction mass became a clear solution and the product immediately formed. The pH value of the reaction mass was above 10. The reaction mass was stirred for 18 hours at 25–30˚C.

[00255] The progress of the reaction was checked by HPLC. A sample of the reaction mass was withdrawn and submitted for IPC-HPLC. Once IPC had been met (IPC-HPLC: 2',3',5'-triacetylazacitidine ≤ 0.5%), the product was filtered under nitrogen, and washed with methanol (300.0 mL) at 25–30˚C. The product was dried at 60–65˚C under vacuum (10–15 mmHg) to give a white to off-white crystalline solid (144.8 g). The average output of 5-azacytidine over multiple runs was about 141.3 g, with an average purity of about 96.8%, and an average yield of about 64.8% (yield based on 5-azacytosine). Over five runs, the maximum yield of 5-azacytidine was about 73.2% (yield based on 5-azacytosine). Over five runs, the maximum HPLC purity of the product was about 98.83%.

4. Reaction Development (Coupling and Deprotection Steps)

[00256] The reaction conditions for the coupling and the deprotection steps were optimized by varying a number of parameters. The results are summarized in **Table 1** below. Inexpensive and commercially readily available metallic Lewis acids, such as stannic chloride and ferric chloride, gave desirable yields for the coupling step.

Table 1: Reaction Development for Coupling and Deprotection

Lewis Acids for Coupling

Example	Lewis Acid	Yield (%)	SOR (°)	HPLC (%)	Remarks
1	TMS-triflate	22.0	+19.3	89.25	
2	Stannic chloride + Zinc chloride (0.6+0.6 eq)	22.9	+34.6	90.93	
3	Boron trifluoride diethyl etherate (1.1 eq)	7.80	+28.9	90.29	
4	Titanium tetrachloride (1.1 eq)	9.17	-	92.10	
5	Stannic chloride + Titanium tetrachloride (0.55+0.55 eq)	18.35	+20.1	89.19	
6	Ferric chloride + TMSCl (1+1 eq)	33.5	+35.2	97.32	
7	Stannic chloride + Zinc chloride (25+75)	-	+30.6	85.6	
8	Stannic chloride	66.4	+26.0	97.30	
9	Ferric chloride	44.8	+19.7	93.28	

Stannic Chloride Mole Ratio (Coupling)

Example	Ratio (Relative to 5-Azacytosine)	Yield (%)	SOR (°)	HPLC (%)	Remarks
10	1.5 eq	23.4	+ 38.4	-	
11	0.7 eq	-	-	-	Reaction not completed
12	1.1 eq	66.4	+26.0	97.30	

1,2,3,5-Tetra-O-acetyl-β-D-ribofuranose Mole Ratio (Coupling)

Example	Ratio (Relative to 5-Azacytosine)	Yield (%)	SOR (°)	HPLC (%)	Remarks
13	0.53 eq	25.7	+ 34.8	97.31	
14	0.9 eq	66.4	+26.0	97.30	

Solvents (Coupling)

Example	Solvent	Yield (%)	SOR (°)	HPLC (%)	Remarks
15	Toluene	-	-	-	Reaction not completed
16	Acetonitrile	41.4	+36.0	97.74	
17	Dichloromethane	66.4	+26.0	97.30	

Temperature (Coupling)

Example	Temp (°C)	Yield (%)	SOR (°)	HPLC (%)	Remarks
18	-5 to –10	41.4	+ 34.2	97.85	Reaction not completed
19	-15 to –20	32.2	+32.5	93.00	
20	0 – 5	66.4	+26.0	97.30	

Base (Hydrolysis)

Example	Base	Yield (%)	SOR (°)	HPLC (%)	Remarks
21	Benzyl trimethyl ammonium hydroxide (0.6 eq)	40.39	+ 27.8	98.99	

5. Preparation of 5-Azacytidine HCl Salt (Salt Formation)

[00257] To remove impurities, such as metal-based impurities, in 5-azacytidine obtained from the deacetylation reaction, a hydrochloride salt of 5-azacytidine was formed. The resulting salt was broken in the next step to give the free base, and the free base was re-crystallized from DMSO by using methanol as anti-solvent to yield the purified 5-azacytidine as the final product.

[00258] To a 3-L 4-neck round bottom flask was added 5-azacytidine (140.0 g, 0.5732 mol, Example A-3) and methanol (1.4 L) at 25–30˚C under nitrogen atmosphere. The suspension was stirred for 10 minutes at 25–30˚C and then cooled to 20–25˚C. Isopropanol-HCl (280.0 mL, ~14% solution) was added slowly at ≤ 25˚C over 5 minutes. After addition, the reaction mass became a clear solution, and after 15–60 minutes, the product was formed. The reaction mass was stirred for a total of 4 hours at 25–30˚C. The product was filtered under nitrogen and washed with methanol (280.0 mL) at 25–30 ˚C. The product was dried at 50–60 ˚C under vacuum (10–15 mmHg) to give the 5-azacytidine mono-hydrochloride salt as white crystalline solid (138.0 g). The average output of 5-azacytidine mono-hydrochloride salt over multiple runs was about 135.5 g, with an average purity of about 99.3%, and an average yield of about 86.4%. Over five runs, the maximum yield of 5-azacytidine mono-hydrochloride salt was about 93.29%. Over five runs, the maximum HPLC purity of the product was about 99.64%.

[00259] In one embodiment, the HCl contents were determined for mono-hydrochloride salts obtained using the above procedure to be the following:

Theoretical: 13.0%

Batch 1A: 12.99%

Batch 1B: 13.95%

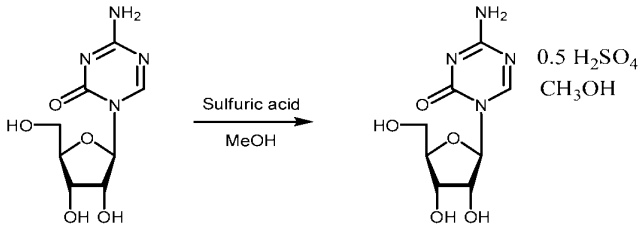
Batch 1C: 12.82%

Batch 1D: 12.66%

Batch 1E: 12.79%.

6. Reaction Development (Salt Formation Step)

(a) Preparation of 5-azacytidine hemisulfate



[00260] A mixture of 5-azacytidine (140.0 g, 0.5732 mol) in methanol (1.4 L) was stirred at 25–30°C under nitrogen atmosphere for 10 minutes and cooled to 20–25°C to give a white slurry. To this mixture was added slowly sulfuric acid (56.16 g, 0.5732 mol) at below 25°C over a period of 30 minutes. The resulting mixture was stirred for 2 hours at 25–30°C. Then the mixture was filtered at 25–30°C under nitrogen and washed with methanol (280 mL). The solid was dried at 50–60°C under vacuum to give a methanol solvate of 5-azacytidine hemisulfate salt as a white solid (163.5 g, MW 325.2; 87.7% yield; melting range: 141.2–144.2°C; SOR: –4.0° (C = 1 in Water at 25°C).

[00261] In one embodiment, the sulfuric acid contents were determined for hemisulfate salt methanol solvates obtained using the above procedure to be the following:

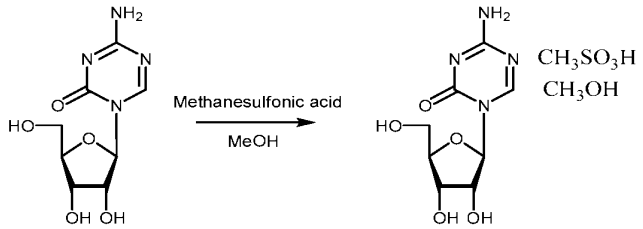
Theoretical: 15.0%

Batch 2A: 15.69%

Batch 2B: 15.67%

Batch 2C: 14.93%.

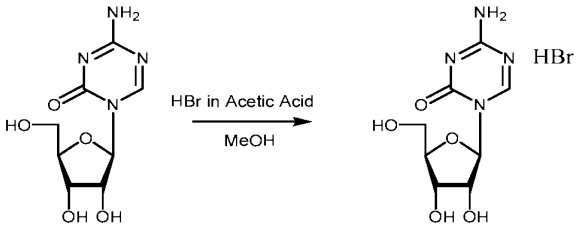
(b) Preparation of 5-azacytidine mesylate



[00262] A mixture of 5-azacytidine (3.0 g, 12.28 mmol) in methanol (30 mL) was stirred at 25–30°C under nitrogen atmosphere for 5 minutes to give a white slurry. To this mixture was added slowly methanesulfonic acid (1.18 g, 12.28 mmol) at below 30°C over a period of 5 minutes to give a clear solution. The resulting mixture was stirred for

2 hours at 25–30°C (after about 15 to 30 minutes, a white solid precipitated). Then the mixture was filtered at 25–30°C under nitrogen and washed with methanol (9 mL). The solid was dried at 50–60°C under vacuum to give a methanol solvate of 5-azacytidine mesylate salt as a white crystalline solid (2.4 g, MW 372.35; 52.7% yield; melting range: 133.5–136.8°C; SOR: –2.3° (C = 1 in Water at 25°C).

(c) Preparation of 5-azacytidine hydrobromide



[00263] A mixture of 5-azacytidine (3.0 g, 12.28 mmol) in methanol (30 mL) was stirred at 25–30°C under nitrogen atmosphere for 5 minutes and cooled to 20–25°C to give a white slurry. To this mixture was added slowly HBr in acetic acid (33% w/w, 3.01 g, 12.28 mmol) at below 25°C over a period of 30 minutes to give a clear solution. The resulting mixture was stirred for 12 hours at 25–30°C (after about 4 to 5 hours, a white solid precipitated). Then the mixture was filtered at 25–30°C under nitrogen and washed with methanol (9 mL). The solid was dried at 50–60°C under vacuum to give 5-azacytidine hydrobromide salt as a white crystalline solid (2.68 g, MW 325.12; 67.0% yield; melting range: 162.4–164.9°C; SOR: –3.9° (C = 1 in Water at 25°C).

[00264] In one embodiment, the HBr contents were determined for hydrobromide salt obtained using the above procedure to be the following:

Theoretical: 24.88%

Batch 3A: 25.15%

Batch 3B: 25.27%.

(d) 5-Azacytidine salts and metal contents

[00265] A number of inorganic and organic acids were evaluated for 5-azacytidine salt formation and the residual metal contents in the 5-azacytidine salts were determined. The results are summarized in **Table 2** below.

[00266] Hydrochloric acid (HCl) formed a stable salt with 5-azacytidine, which had a low content of tin (8 ppm). The formation of 5-azacytidine hydrochloride salt afforded products that were substantially free from residual metal impurities.

Table 2: Reaction Development for Salt Formation

Example #	Acid	Yield (%)	SOR (°)	MR (°C)	HPLC (%)	Sn (ppm)	Remarks
1	Hydrochloric acid	85.0	-4.0	162.1-164.1	82.68	8.0	Isolated the 5-azacytidine HCl salt
2	Hydrobromic acid in acetic acid	67.0	-	162.4-164.9	89.71	240.0	Isolated the 5-azacytidine HBr salt
3	Sulfuric acid	88.1	-3.0	145.2-148.8	92.98	-	Isolated the 5-azacytidine hemisulfate salt
4	Methanesulfonic acid	52.7	-2.3	133.5-136.8	98.06	-	Isolated the 5-azacytidine mesylate salt

7. Preparation of Free Base of 5-Azacytidine (Free Base Formation)

[00267] To a 3-L 4-neck round bottom flask was added 5-azacytidine HCl salt (120.0 g, 0.4274 mol, Example A-5) and methanol (1.2 L) at 25–30˚C under nitrogen atmosphere. The suspension was stirred for 10 minutes at 25–30˚C. Triethylamine (64.8 g, 0.64 mol) was added slowly at ≤ 30˚C. The slurry was stirred for 2 hours at 25–30˚C. The product was filtered under nitrogen and washed with methanol (300 mL) at 25–30˚C. The presence of chloride in the filtrate was monitored by adding 10% silver nitrate solution to a fraction of the filtrate. The test showed the presence of chloride at this stage (white turbidity observed). The wet product was suspended in methanol (1.0 L) and stirred for 10 minutes at 25–30˚C. The product was filtered under nitrogen and washed with methanol (200.0 mL) at 25–30˚C. No chloride was detected in the filtrate after the methanol wash. The product was then dried at 50–60˚C under vacuum (10–15 mmHg) to give the 5-azacytidine free base as a white crystalline solid (103.5 g). The average output of 5-azacytidine free base over multiple runs was about 103.4 g, with an average purity of about 99.5%, and an average yield of about 97.5%. Over five runs, the maximum yield of 5-azacytidine free base was about 99.13%. Over five runs, the maximum HPLC purity of the product was about 99.77%.

8. Reaction Development (Free Base Formation Step)

[00268] A number of bases were evaluated for the salt breaking and 5-azacytidine free base formation step. The results are summarized in **Table 3** below.

Table 3: Reaction Development for Salt Breaking/Free Base Formation

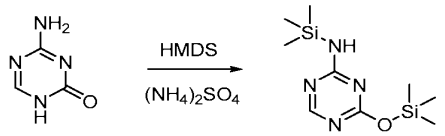
Example #	Base	Yield (%)	SOR (°)	MR (°C)	HPLC (%)
1	Diisopropylethyl amine	97.7	+37.1	219.7-221.6	99.30
2	Pyridine	91.9	+23.0	165.3-168.6	99.54
3	Diisopropyl amine	94.2	+35.5	217.2-219.4	99.46
4	2,6-Lutidine	96.5	+23.3	167.4-170.9	99.47
5	N-Methyl morpholine	100	+36.0	217.2-219.4	99.23
6	N,N-Dicyclohexyl methyl amine	96.5	+36.6	219.5-221.9	99.22

9. Re-Crystallization of 5-Azacytidine

[00269] To a 500-mL 4-neck round bottom flask was added 5-azacytidine free base (80.0 g, 0.3275 mol, Example A-7) and DMSO (200.0 mL) at 25–30˚C under nitrogen atmosphere. The suspension was stirred for 10 minutes at 25–30˚C, and gradually heated to 85–90˚C. The mass was stirred for 10 minutes at 85–90˚C to get a clear solution. The clear solution was filtered through a filter paper to remove insoluble particles at 85–90˚C, and washed with hot DMSO (80.0 mL). Methanol (1.2 L) was slowly charged to the filtered DMSO solution containing the product at 70–80˚C over a period of 3–4 hours. The mixture was stirred for 15 minutes at 70–80˚C, and gradually cooled to 25–30˚C over 2–3 hours. The mass was stirred for 15 hours at 25–30˚C, and filtered under nitrogen. The solid product was washed with methanol (240.0 mL) at 25–30˚C. The solid product was dried at 85–90˚C under vacuum (10–15 mmHg) until the loss on drying (LOD) fell below 0.4% to provide 5-azacytidine as a white crystalline solid (75.6 g). The average output of 5-azacytidine after the re-crystallization step over multiple runs was about 75.8 g, with an average purity of about 99.6%, and an average yield of about 95.1%. Over three runs, the maximum yield of 5-azacytidine was about 95.80%. Over three runs, the maximum HPLC purity of the product was about 99.71%.

B. Ferric Chloride Route

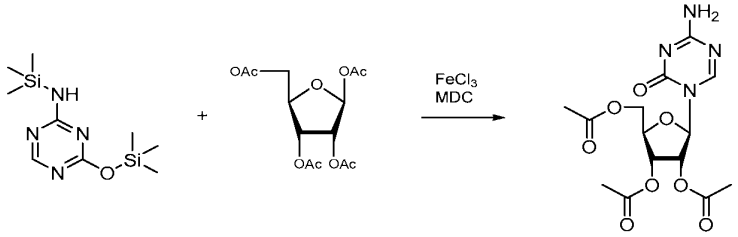
1. Preparation of Silylated 5-Azacytosine



[00270] 5-Azacytosine (200.0 g, 1.7842 mol, purity ≥ 98%) and hexamethyldisilazane (HMDS) (1.4 L, 6.72 mol, purity ≥ 98%) were charged into a 3-L 4-neck round bottom flask at 25–30˚C under nitrogen atmosphere. Ammonium sulfate (10.0 g, 0.0756 mol) was added. The mixture was gradually heated to reflux at 125–130˚C. The reflux was maintained for 6 hours. Typically, the reaction mass became a clear solution after 2–4 hours, and the reaction was substantially complete as soon as the clear solution was formed.

[00271] The reaction mass was gradually cooled to 40–50˚C. HMDS was distilled off at 40–50˚C under vacuum (10–15 mmHg) to give a white solid. Nitrogen was used to break the vacuum over the solid. Toluene (400.0 mL) was added to the solid residue at 25–30˚C, and the solvent was distilled off at 40–50˚C under vacuum (10–15 mmHg) to yield a solid. Nitrogen was used to break the vacuum over the solid. The solid was gradually cooled to 25–30˚C, and carried through to the next step. HMDS was recovered in 75–80% yield with a purity of about 90–95%.

2. Preparation of Acetyl-Protected 5-Azacytidine (Coupling)



[00272] To silylated 5-azacytosine (Example B-1) was added dichloromethane (2.0 L) at 25–30˚C under nitrogen atmosphere. The mixture was stirred for 10 minutes at 25–30˚C to obtain a clear solution.

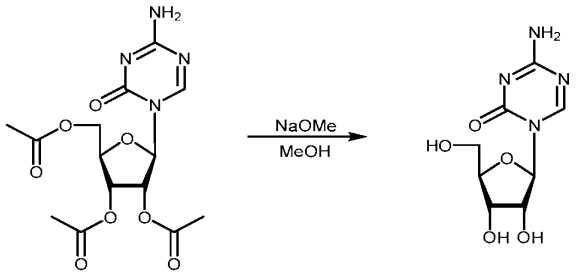
[00273] In a separate 5-L 4-neck round bottom flask under nitrogen was added 1,2,3,5-tetra-O-acetyl-β-D-ribofuranose (511.1 g, 1.6058 mol) and dichloromethane (1.0 L) at 25–30˚C. The mixture was stirred for 10 minutes at 25–30˚C to obtain a clear solution. Anhydrous ferric chloride (361.7 g, 1.9627 mol) was added in one lot at 25–30˚C. The

mixture was stirred for 10 minutes at 25–30˚C to obtain a dark red mass. The reaction mass was gradually cooled to 0–5˚C. Silylated 5-azacytosine (Example B-1) in dichloromethane was added drop-wise at ≤ 10˚C over 90 minutes (addition slightly exothermic), and a clear dark brown mass was formed. The reaction mass was stirred for 15 hours at 0–5˚C under nitrogen atmosphere.

[00274] The progress of the reaction was checked by HPLC. 5 g of reaction mass was withdrawn and neutralized with saturated aqueous $NaHCO_3$ solution at 10˚C. The dichloromethane layer was separated and submitted for IPC-HPLC. Once IPC had been met (5-azacytosine no more than 0.5%), the reaction mass was transferred to a 10-L round bottom flask for work up.

[00275] To the reaction mass was added dichloromethane (3.0 L) and sodium bicarbonate (2.0 kg) at ≤ 10˚C. Chilled water (1.6 L) was added drop-wise at ≤ 10˚C over 60 minutes (addition exothermic). The reaction mass was dark brown and had a final pH of ~6. The mixture was stirred for 30 minutes at ≤ 10˚C. After 15–30 minutes, brown solid of ferric oxide settled to the bottom of the flask. Hyflo® (200 g) was added to the mixture, and the mass was stirred for 10 minutes at ≤ 10˚C. The mixture was filtered through Hyflo®, and washed with dichloromethane (1.0 L). The organic layer was separated at ≤ 10˚C, and washed with water (1.0 L) at ≤ 10˚C. The organic layer was then washed with 10% EDTA disodium salt solution twice (300 g salt, 2 x 1.5 L) and water once (1.0 L) at ≤ 10˚C. The organic layer was dried over anhydrous sodium sulfate, and the solvent was distilled off at 40–45˚C under atmospheric conditions, and further dried at 40–45˚C under vacuum (10–15 mmHg) to give a sticky foaming solid. To the residue was added methanol (200 mL) at 30–35˚C. The solvent was then distilled off at 40–45˚C under vacuum (10–15 mmHg), and degassed under vacuum for 30 minutes to afford 2',3',5'-triacetyl-5-azacytidine as a sticky foaming white solid. The average output of 2',3',5'-triacetyl-5-azacytidine over multiple runs was about 524 g, with an average purity of about 63.3%, and an average yield of about 50.2% (% yield takes into account the HPLC purity of the product). Over three runs, the maximum yield of 2',3',5'-triacetyl-5-azacytidine was about 64.9% (% yield takes into account the HPLC purity of the product). Over three runs, the maximum HPLC purity of the product was about 82.45%.

3. Preparation of 5-Azacytidine (Deprotection)



[00276] To 2',3',5'-triacetyl-5-azacytidine (Example B-2) was added methanol (2.0 L) at 25–30˚C. The slurry was stirred for 10 minutes at 25–30˚C, and 25% sodium methoxide in methanol (80.0 mL) was added slowly at 25–30˚C under nitrogen atmosphere. After addition, the reaction mass became a clear solution and the product immediately formed. The pH value of the reaction mass was above 10. The reaction mass was stirred for 18 hours at 25–30˚C.

[00277] The progress of the reaction was checked by HPLC. A sample of the reaction mass was withdrawn and submitted for IPC-HPLC. Once IPC had been met (IPC-HPLC: 2',3',5'-triacetylazacitidine ≤ 0.5%), the product was filtered under nitrogen, and washed with methanol (600.0 mL) at 25–30˚C. The product was dried at 60–65˚C under vacuum (10–15 mmHg) to give an off-white solid (195.5 g). The average output of 5-azacytidine over multiple runs was about 193.7 g, with an average purity of about 91.8%, and an average yield of about 44.4% (based on 5-azacytosine). Over three runs, the maximum yield of 5-azacytidine was about 44.86% (based on 5-azacytosine). Over three runs, the maximum HPLC purity of the product was about 93.28%.

4. Reaction Development (Coupling and Deprotection Steps)

[00278] The reaction conditions for the coupling step were optimized by varying parameters such as the molar ratio of the reagents. The results are summarized in the **Table 4** below.

Table 4: Reaction Development for the Ferric Chloride Coupling Step

Ferric Chloride Mole Ratio (Coupling)

Example #	Ratio (Relative to Azacytosine)	Yield (%)	SOR (°)	HPLC (%)	Remarks
1	1.5 eq	33.0	+ 31.4	95.46	
2	1.1 eq	44.8	+ 19.7	93.28	

5. Preparation of 5-Azacytidine HCl Salt (Salt Formation)

[00279] To a 3-L 4-neck round bottom flask was added 5-azacytidine (175.0 g, 0.7166 mol, Example B-3) and methanol (1.75 L) at 25–30˚C under nitrogen atmosphere. The suspension was stirred for 10 minutes at 25–30˚C and then cooled to 20–25˚C. Isopropanol-HCl (350.0 mL, ~14% solution) was added slowly at ≤ 25˚C over 5 minutes. After addition, the reaction mass became a clear solution, and after 15–60 minutes, the product was formed. The reaction mass was stirred for a total of 4 hours at 25–30˚C. The product was filtered under nitrogen and washed with methanol (350.0 mL) at 25–30˚C. The product was dried at 50–60˚C under vacuum (10–15 mmHg) to give the 5-azacytidine mono-hydrochloride salt as off-white crystalline solid (151.0 g). The average output of 5-azacytidine mono-hydrochloride salt over multiple runs was about 148.8 g, with an average purity of about 99.0%, and an average yield of about 74.0%. Over three runs, the maximum yield of 5-azacytidine mono-hydrochloride salt was about 75.09%. Over three runs, the maximum HPLC purity of the product was about 99.12%.

6. Preparation of Free Base of 5-Azacytidine (Free Base Formation)

[00280] To a 3-L 4-neck round bottom flask was added 5-azacytidine hydrochloride salt (130.0 g, 0.4631 mol, Example B-5) and methanol (1.3 L) at 25–30˚C under nitrogen atmosphere. The suspension was stirred for 10 minutes at 25–30˚C. Triethylamine (70.3 g, 0.6946 mol) was added slowly at ≤ 30˚C. The slurry was stirred for 2 hours at 25–30˚C. The product was filtered under nitrogen and washed with methanol (300.0 mL) at 25–30˚C. The presence of chloride in the filtrate was checked by adding 10% silver nitrate solution to a fraction of the filtrate. The test showed the presence of chloride at this stage (white turbidity observed). The wet product was suspended in methanol (1.0 L) and stirred for 10 minutes at 25–30˚C. The product was filtered under nitrogen and washed with methanol (200.0 mL) at 25–30˚C. No chloride was detected in the filtrate after the methanol wash. The product was then dried at 50–60˚C under vacuum (10–15 mmHg) to give the 5-azacytidine free base as an off-white solid (113.0 g). The average output of 5-azacytidine free base over multiple runs was about 112.5 g, with an average purity of about 99.2%, and an average yield of about 99.5%. Over three runs, the maximum yield of 5-azacytidine free base was about 99.99%. Over three runs, the maximum HPLC purity of the product was about 99.38%.

7. Re-Crystallization of 5-Azacytidine

[00281] To a 500-mL 4-neck round bottom flask was added 5-azacytidine free base (100.0 g, 0.4095 mol, Example B-6) and DMSO (250.0 mL) at 25–30˚C under nitrogen atmosphere. The suspension was stirred for 10 minutes at 25–30˚C, and gradually heated to 85–90˚C. The mass was stirred for 10 minutes at 85–90˚C to obtain a clear solution. The clear solution was filtered through a filter paper to remove insoluble particles at 85–90˚C, and washed with hot DMSO (100.0 mL). Methanol (1.5 L) was slowly charged to the filtered DMSO solution containing the product at 70–80˚C over a period of 3–4 hours. The mixture was stirred for 15 minutes at 70–80˚C, and gradually cooled to 25–30˚C over 2–3 hours. The mass was stirred for 15 hours at 25–30˚C, and filtered under nitrogen. The solid product was washed with methanol (240.0 mL) at 25–30˚C. The solid product was dried at 85–90˚C under vacuum (10–15 mmHg) until the LOD fell below 0.4% to provide 5-azacytidine as a white solid (86.0 g). The average output of 5-azacytidine after the re-crystallization step over multiple runs was about 86.0 g, with an average purity of about 99.4%, and an average yield of about 86.0%. Over three runs, the maximum yield of 5-azacytidine was about 87.5%. Over three runs, the maximum HPLC purity of the product was about 99.54%.

C. Comparative Data

[00282] Reaction yields, purity profiles (by HPLC), water contents (by KF, % w/w), specific optional rotation (SOR, $[\alpha]_D$), and metal impurity contents (*e.g.*, Sn or Fe, when applicable) were gathered from repeat batches of various steps along three different routes used for preparing 5-azacytidine. The triflate route utilized TMS-triflate as the Lewis acid in the coupling step. The stannic chloride route utilized stannic chloride as the Lewis acid in the coupling step (*See* Example A herein). The ferric chloride route utilized ferric chloride as the Lewis acid in the coupling step (*See* Example B herein). The following tables summarize the data for specific batches. Both stannic chloride and ferric chloride gave good overall yield for the synthesis of 5-azacytidine from 5-azacytosine and acetyl protected sugar. Hydrochloride salt formation and the subsequent steps of breaking the salt to form the free base and re-crystallization of the free base consistently provided 5-azacytidine batches that were substantially free of metal impurities.

1. Preparation of Triacetyl 5-Azacytidine

[00283] **Table 5**: Triflate Route

Example #	Input (g)*	Output (g)†	Yield (%)‡	HPLC Purity (RRT, A%)								
				1.00	1.06	0.52	0.71	0.84	0.91	0.94	0.95	1.14
1	10.0	25.8	29.1	37.34	22.32	1.95	0.18	3.58	0.84	1.15	9.20	8.60
2	10.0	24.5	22.7	30.72	17.49	2.59	0.77	3.45	0.64	0.94	9.40	7.35

[00284] **Table 6**: Stannic Chloride Route

Example #	Input (g)*	Output (g)†	Yield (%)‡	HPLC Purity (RRT, A%)								
				1.00	1.06	0.52	0.71	0.84	0.91	0.94	0.95	1.14
3	100	278.0	72.3	85.94	8.52	-	-	-	0.33	1.21	0.40	-
4	100	308.0	81.8	87.8	-	-	-	-	0.54	-	0.69	0.12
5	100	310.0	78.1	83.30	15.37	-	-	-	0.42	-	-	0.26
6	100	315.0	75.0	78.70	7.01	1.59	6.33	3.17	-	-	-	-
7	100	315.0	78.9	82.75	5.49	0.69	3.58	3.01	-	-	-	-

[00285] **Table 7**: Ferric Chloride Route

Example #	Input (g)*	Output (g)†	Yield (%)‡	HPLC Purity (RRT, A%)						
				1.00	1.06	0.82	0.83	0.95	0.96	1.41
8	200.0	520.0	64.9	82.45	8.84	0.64	1.45	-	0.14	2.20
9	200.0	535.0	50.6	62.54	0.15	3.73	6.38	1.61	0.64	5.84
10	200.0	517.0	35.1	44.85	2.03	11.99	8.34	4.02	3.29	3.87

* 5-azacytosine
† Residue weight
‡ The % yield by theory is based on HPLC purity.

2. Preparation of 5-Azacytidine by Deacetylation

[00286] **Table 8**: Triflate Route

Example #	Input (g)*	Output (g)	Yield (%)‡	HPLC (%)	KF (%)	SOR (°)
1	10.0	5.5	25.2	93.05	1.11	+ 28.6
2	10.0	4.8	22.0	89.25	1.65	+19.29

[00287] **Table 9**: Stanic Chloride Route

Example #	Input (g)*	Output (g)	Yield (%)‡	HPLC (%)	KF (%)	SOR (°)	Sn (ppm)
3	100.0	132.0	60.58	98.14	0.49	+28.9	2.22
4	100.0	137.5	63.11	95.17	1.54	+25.6	<1.0
5	100.0	132.7	60.90	98.83	1.08	+27.5	46.9
6	100.0	144.8	66.46	97.30	1.40	+26.0	118.22
7	100.0	159.5	73.20	94.42	1.11	+28.6	144.38

[00288] **Table 10**: Ferric Chloride Route

Example #	Input (g)*	Output (g)	Yield (%)‡	HPLC (%)	KF (%)	SOR (°)	Fe (ppm)
8	200.0	195.5	44.86	93.28	2.09	+19.7	77.1
9	200.0	190.0	43.60	90.48	2.14	+19.6	36.9
10	200.0	195.5	44.86	91.73	2.03	+24.1	49.1

* 5-azacytosine
‡ The % yield is based on 5-azacytosine

3. Preparation of 5-Azacytidine Hydrochloride Salt

[00289] **Table 11**: Stannic Chloride Route

Example #	Input (g)	Output (g)	Yield (%)	HPLC (%)	KF (%)	SOR (°)	Sn (ppm)
1	140.0	138.0	85.79	99.57	0.24	-5.4	1.62
2	140.0	133.0	82.68	99.48	0.32	-4.5	2.75
3	95.0	87.8	80.43	98.47	0.49	-4.5	<1.0
4	125.0	134.0	93.29	99.64	0.49	-5.2	<1.0
5	130.0	134.0	89.71	99.52	1.54	-4.5	<1.0

[00290] **Table 12**: Ferric Chloride Route

Example #	Input (g)	Output (g)	Yield (%)	HPLC (%)	KF (%)	SOR (°)	Fe (ppm)
6	175.0	151.0	75.09	99.12	0.21	-3.6	<1.0
7	175.0	146.0	72.61	98.99	0.21	-3.8	<1.0
8	175.0	149.5	74.35	98.89	0.52	-3.9	<1.0

4. Preparation of 5-Azacytidine Free Base from HCl Salt

[00291] **Table 13**: Stannic Chloride Route

Example #	Input (g)	Output (g)	Yield (%)	HPLC (%)	KF (%)	SOR (°)	Sn (ppm)
1	120.0	103.5	99.13	99.48	0.54	+34.7	1.8
2	120.0	103.3	98.84	99.67	0.32	+33.3	2.5
3	80.0	66.6	95.68	99.00	0.53	+35.7	<1.0
4	85.0	71.3	96.41	99.77	0.25	+36.5	<1.0
5	125.0	105.8	97.28	99.72	0.11	+35.8	<1.0

[00292] **Table 14**: Ferric Chloride Route

Example #	Input (g)	Output (g)	Yield (%)	HPLC (%)	KF (%)	SOR (°)	Fe (ppm)
6	130.0	113.0	99.99	99.04	0.61	+36.6	3.11
7	130.0	112.5	99.47	99.38	0.63	+36.5	3.02
8	130.0	112.0	99.02	99.21	0.59	+36.6	<1.0

5. Re-Crystallization of 5-Azacytidine

[00293] **Table 15**: Stannic Chloride Route

Exmp. #	Yield (%)	SOR (°)	HPLC Purity @ 210 nm (RRT, A%)						HPLC Assay	Chemical Assay	Sn	KF	LOD
			1.00	0.68	3.5	4.6	5.2	Total	% w/w	% w/w	(ppm)	(%)	(%)
1	94.50	+37.7	99.71	0.11	0.18	-	-	0.29	100.33	98.51	<1.0	0.59	0.09
2	95.00	+38.2	99.61	0.02	0.25	0.10	0.02	0.39	99.34	99.85	<1.0	0.48	0.29
3	95.80	+37.3	99.48	0.09	0.23	0.10	0.10	0.52	100.35	99.71	<1.0	0.26	0.10

[00294] **Table 16**: Ferric Chloride Route

Exmp. #	Yield (%)	SOR (°)	HPLC Purity @ 210 nm (RRT, A%)						HPLC Assay	Chemical Assay	Fe	KF	LOD
			1.00	0.68	3.5	4.6	5.2	Total	% w/w	% w/w	(ppm)	(%)	(%)
4	86.0	+39.7	99.54	0.03	0.29	0.11	0.03	0.46	98.25	99.13	<1.0	0.14	0.20
5	87.5	+38.2	99.37	0.11	0.34	0.15	0.03	0.63	99.42	98.33	<1.0	0.28	0
6	84.6	+38.2	99.47	0.03	0.28	0.20	0.02	0.53	98.71	98.84	<1.0	0.48	0.20

D. Analytical Data

[00295] The analytical data were obtained and provided below for repeat batches of 5-azacytidine final products, salts of 5-azacytidine, and the triacetyl 5-azacytidine intermediate.

1. 5-Azacytidine Final Product

[00296] ^{1}H NMR (DMSO-d_6) δ 8.57 (s, 1H), 7.52 (d, 2H, J = 10.6 Hz), 5.65 (d, 1H, J = 3.7 Hz), 5.42 (d, 1H, J = 5.1 Hz), 5.10 (t, 1H, J = 5.0 Hz), 5.02 (d, 1H, J = 5.7 Hz), 4.07 (m, 1H), 4.00 (m, 1H), 3.84 (m, 1H), 3.67 (m, 1H), 3.55 (m, 1H).

[00297] ^{13}C NMR (DMSO-d_6) δ 166.1, 156.7, 153.7, 89.6, 84.6, 74.2, 69.3, 60.5.

[00298] LC-MS ESI: m/z 245.2 ($M + H^+$), 267.1 ($M + Na^+$).

[00299] For 5-azacytidine obtained from the stannic chloride route, the IR spectrum is shown in **Figure 1**; the DSC plot is shown in **Figure 2**; the XRPD pattern is shown in **Figure 3**. The XRPD data is further provided in the tables below:

Table 17: XRPD - Strongest 3 peaks

no.	Peak no.	2Theta (deg)	d (A)	I/I1	FWHM (deg)	Intensity (Counts)	Integrated Int (Counts)
1	12	22.9979	3.86406	100	0.26900	1955	32423
2	4	12.9834	6.81324	94	0.23960	1843	22060
3	13	23.8179	3.73285	87	0.28000	1708	26470

Table 18: XRPD - Peak Data List

Peak no.	2Theta (deg)	d (A)	I/I1	FWHM (deg)	Intensity (Counts)	Integrated Int (Counts)
1	11.8200	7.48110	11	0.17140	210	2859
2	12.1412	7.28390	75	0.25280	1471	18956
3	12.6200	7.00860	35	0.29760	681	11254
4	12.9834	6.81324	94	0.23960	1843	22060
5	13.9400	6.34777	5	0.17340	102	2226
6	14.3503	6.16719	77	0.25620	1509	20932
7	16.4330	5.38996	69	0.23430	1340	18847
8	18.6020	4.76609	50	0.30420	982	15985
9	18.9777	4.67257	47	0.31320	927	14624
10	20.1354	4.40646	22	0.26230	432	6317
11	21.2857	4.17085	35	0.24720	679	10220
12	22.9979	3.86406	100	0.26900	1955	32423
13	23.8179	3.73285	87	0.28000	1708	26470
14	24.4956	3.63109	4	0.29530	74	1696
15	25.3727	3.50752	11	0.27200	208	3112
16	26.1000	3.41141	10	0.32880	187	4032
17	26.8000	3.32387	32	0.24100	633	7938
18	27.0310	3.29599	74	0.38780	1448	21079
19	27.6000	3.22932	6	0.22320	122	1731
20	29.2077	3.05512	54	0.40890	1059	21215
21	29.5800	3.01751	29	0.28080	572	8109
22	30.0600	2.97041	12	0.40400	233	4726
23	30.3400	2.94363	22	0.27600	439	5627
24	31.9890	2.79555	26	0.33460	518	9593
25	32.8941	2.72067	24	0.48740	466	12216
26	33.5800	2.66665	13	0.29640	245	4483
27	35.6631	2.51552	5	0.40630	100	2420
28	36.7600	2.44293	4	0.42220	76	2237
29	37.1600	2.41755	4	0.00000	79	0
30	37.4800	2.39764	7	0.00000	142	0
31	37.7800	2.37929	9	0.36000	167	3804
32	38.5538	2.33330	9	0.57560	178	5997
33	40.6547	2.21744	4	0.21940	71	1081
34	41.6215	2.16813	6	0.27300	114	2437
35	43.2476	2.09031	23	0.50800	459	13218
36	43.9000	2.06074	3	0.20000	61	953
37	45.4727	1.99306	6	0.38550	113	2474
38	45.9450	1.97366	7	0.26140	130	1753
39	46.4544	1.95320	3	0.23880	59	823
40	48.5714	1.87290	3	0.21710	59	899

[00300] For 5-azacytidine obtained from the ferric chloride route, the IR spectrum is shown in **Figure 4**; the DSC plot is shown in **Figure 5**; the XRPD pattern is shown in **Figure 6**. The XRPD data is further provided in the tables below.

Table 19: XRPD - Strongest 3 peaks

no.	Peak no.	2Theta (deg)	d (A)	I/I1	FWHM (deg)	Intensity (Counts)	Integrated Int (Counts)
1	12	22.9372	3.87415	100	0.28580	1875	33155
2	16	26.9754	3.30265	77	0.39750	1453	29755
3	13	23.7666	3.74079	75	0.28730	1411	22685

Table 20: XRPD - Peak Data List

Peak no.	2Theta (deg)	d (A)	I/I1	FWHM (deg)	Intensity (Counts)	Integrated Int (Counts)
1	6.2567	14.11507	3	0.21750	64	900
2	12.1042	7.30608	30	0.29350	568	9641
3	12.5625	7.04055	43	0.26670	803	10544
4	12.9293	6.84163	40	0.25960	748	10118
5	14.2979	6.18967	39	0.27110	729	11550
6	16.0000	5.53483	4	0.13000	78	1032
7	16.3803	5.40718	44	0.24810	822	11165
8	18.5800	4.77168	37	0.37060	697	12661
9	18.9199	4.68672	49	0.31740	923	13819
10	20.0734	4.41993	20	0.28210	374	5876
11	21.2364	4.18042	29	0.26330	552	8712
12	22.9372	3.87415	100	0.28580	1875	33155
13	23.7666	3.74079	75	0.28730	1411	22685
14	25.3226	3.51435	15	0.27870	276	4329
15	26.0600	3.41655	4	0.28880	69	1818
16	26.9754	3.30265	77	0.39750	1453	29755
17	27.5400	3.23622	6	0.26660	105	1838
18	29.2000	3.05590	30	0.46500	567	12422
19	29.5200	3.02350	24	0.32000	454	8137
20	30.0000	2.97621	11	0.00000	208	0
21	30.2800	2.94932	24	0.29420	441	8207
22	31.9369	2.79999	28	0.33290	529	10079
23	32.7835	2.72959	22	0.40900	411	9358
24	33.5200	2.67128	10	0.30660	184	3603
25	35.6416	2.51698	6	0.34330	106	2269
26	37.1600	2.41755	3	0.29000	59	1190
27	37.4200	2.40135	7	0.37000	123	1475
28	37.7200	2.38293	4	0.42400	80	1526
29	38.4710	2.33813	8	0.56870	151	4430
30	41.5706	2.17067	7	0.28990	134	2135
31	41.9600	2.15142	3	0.30000	62	1080
32	43.2083	2.09212	27	0.49400	497	13585
33	43.9525	2.05840	4	0.19640	79	920
34	45.3612	1.99770	7	0.43750	126	2877
35	45.8820	1.97623	4	0.30800	78	1268
36	48.5080	1.87520	4	0.31200	71	1792

[00301] Additional analytical data for repeat batches of the 5-azacytidine final product are provided in the tables below.

[00302] **Table 21:** Stannic Chloride Route

Exmp. #	LOD at 105±5°C (%w/w)	Residue on ignition (%w/w)	$[\alpha]_D$ (1.0% water) (in°)	Heavy metal (ppm)	Tin content (ppm)	DSC	HPLC single impurity %area	HPLC total impurity %area	Assay by HPLC %w/w	Assay by chemical %w/w
1	0.09	0.13	+37.7	<20	<1.0	234.3°	0.25	0.39	100.33	98.51
2	0.29	0.13	+38.2	<20	<1.0	234.2°	0.30	0.37	99.34	99.85
3	0.10	0.08	+37.3	<20	<1.0	238.9°	0.27	0.42	100.35	99.71

[00303] **Table 22:** Ferric Chloride Route

Exmp. #	LOD at 105±5°C (%w/w)	Residue on ignition (%w/w)	$[\alpha]_D$ (1.0% water) (in°)	Heavy metal (ppm)	Iron content (ppm)	DSC	HPLC single impurity %area	HPLC total impurity %area	Assay by HPLC %w/w	Assay by chemical %w/w
4	0.20	0.02	+39.7	<20	<1.0	238.3°	0.37	0.47	98.25	99.13
5	Nil	0.09	+38.2	<20	<1.0	235.2°	0.39	0.59	99.42	98.33
6	0.20	0.09	+37.9	<20	<1.0	238.5°	0.30	0.40	98.71	98.84

[00304] The metal content of repeat batches of 5-azacytidine was determined. For 5-azacytidine obtained from the stannic chloride route, the tin contents in three repeat batches of 5-azacytidine final product were <0.1 ppm, <0.1 ppm, and 0.14 ppm, respectively. For 5-azacytidine obtained from the ferric chloride route, the iron contents in three repeat batches of 5-azacytidine final product were <0.1 ppm, <0.1 ppm, and <0.1 ppm, respectively.

2. 5-Azacytidine Mono-Hydrochloride Salt

[00305] ^{1}H NMR (DMSO-d_6) δ 9.86 (s, 1H), 8.94 (s, 2H), 8.91 (s, 1H), 5.58 (d, 1H, J = 1.4 Hz), 4.12 (m, 1H), 4.05 (m, 1H), 3.90 (m, 1H), 3.76 (m, 1H), 3.59 (m, 1H).

[00306] ^{13}C NMR (DMSO-d_6) δ 160.3, 158.3, 146.6, 90.7, 84.5, 74.2, 68.0, 59.2.

[00307] LC-MS ESI: m/z 245.2 ($M + H^+$).

[00308] The IR spectrum of 5-azacytidine mono-hydrochloride salt is shown in **Figure 7**; the DSC plot of 5-azacytidine mono-hydrochloride salt is shown in **Figure 8**. For 5-azacytidine mono-hydrochloride salt obtained from the stannic chloride route, the XRPD pattern is shown in **Figure 9**. For 5-azacytidine mono-hydrochloride salt obtained from the ferric chloride route, the XRPD pattern is shown in **Figure 10**. The XRPD data are further provided in the tables below:

Table 23: XRPD (Stannic Chloride Route) – Strongest 3 peaks

no.	Peak no.	2Theta (deg)	d (A)	I/I1	FWHM (deg)	Intensity (Counts)	Integrated Int (Counts)
1	10	22.4151	3.96319	100	0.16700	2575	23714
2	19	29.6437	3.01117	70	0.17140	1806	16497
3	1	10.1820	8.68063	38	0.15390	967	9694

Table 24: XRPD (Stannic Chloride Route) – Peak Data List

Peak no.	2Theta (deg)	d (A)	I/I1	FWHM (deg)	Intensity (Counts)	Integrated Int (Counts)
1	10.1820	8.68063	38	0.15390	967	9694
2	13.5600	6.52479	3	0.16000	81	1615
3	13.8880	6.37142	34	0.15420	886	7580
4	17.2906	5.12450	32	0.17580	836	8843
5	18.5819	4.77120	12	0.17910	298	3115
6	19.4600	4.55784	4	0.10820	102	912
7	19.6480	4.51465	19	0.17090	495	4403
8	21.0139	4.22418	16	0.15630	406	3852
9	22.1200	4.01539	6	0.14120	163	2682
10	22.4151	3.96319	100	0.16700	2575	23714
11	23.3341	3.80914	32	0.17430	822	8661
12	24.9266	3.56927	18	0.18980	464	5140
13	25.9609	3.42937	13	0.14690	342	2980
14	26.7005	3.33603	11	0.16860	290	2812
15	27.5877	3.23073	9	0.17970	230	2320
16	28.7607	3.10157	22	0.17130	556	5415
17	29.2000	3.05590	3	0.11360	80	557
18	29.3400	3.04164	10	0.15880	269	2565
19	29.6437	3.01117	70	0.17140	1806	16497
20	29.9278	2.98323	16	0.18500	416	4216
21	30.3686	2.94092	3	0.19730	83	862
22	31.0303	2.87970	3	0.18460	87	1019
23	32.2457	2.77388	22	0.16050	565	5201
24	34.1244	2.62534	21	0.23380	549	7173
25	35.0000	2.56164	3	0.18660	81	1120
26	35.1400	2.55175	5	0.25500	121	1271
27	38.9800	2.30876	4	0.11600	104	619
28	39.1250	2.30054	9	0.25000	239	2803
29	40.8414	2.20773	5	0.18940	138	1917
30	41.8511	2.15677	4	0.20630	102	1137
31	42.2800	2.13588	3	0.15140	80	1171
32	42.5000	2.12533	3	0.00000	83	0
33	42.7600	2.11301	6	0.19640	165	1745
34	42.9000	2.10643	5	0.16200	122	940
35	43.2009	2.09246	3	0.24180	77	949
36	44.0600	2.05363	4	0.21240	104	1331
37	44.1800	2.04833	5	0.00000	125	0
38	44.3800	2.03956	7	0.00000	180	0
39	44.6000	2.03001	4	0.00000	95	0
40	44.8044	2.02122	6	0.18660	153	1761
41	45.2044	2.00426	3	0.20520	86	927

Table 25: XRPD (Ferric Chloride Route) – Strongest 3 peaks

no.	Peak no.	2Theta (deg)	d (A)	I/I1	FWHM (deg)	Intensity (Counts)	Integrated Int (Counts)
1	9	22.4639	3.95469	100	0.16700	2537	23682
2	17	29.6916	3.00642	65	0.17360	1647	14121
3	3	13.9291	6.35272	36	0.16820	916	9501

Table 26: XRPD (Ferric Chloride Route) – Peak Data List

Peak no.	2Theta (deg)	d (A)	I/I1	FWHM (deg)	Intensity (Counts)	Integrated Int (Counts)
1	9.8800	8.94528	3	0.18000	77	1671
2	10.2256	8.64371	34	0.16640	870	7850
3	13.9291	6.35272	36	0.16820	916	9501
4	17.3347	5.11156	34	0.18500	851	9333
5	18.6294	4.75914	13	0.17450	326	3241
6	19.6938	4.50426	23	0.17410	585	5889
7	21.0636	4.21433	18	0.15630	447	4150
8	22.1400	4.01181	4	0.13200	93	1691
9	22.4639	3.95469	100	0.16720	2537	23682
10	23.3813	3.80156	34	0.17920	874	9272
11	24.9741	3.56259	18	0.17900	465	4759
12	26.0052	3.42363	13	0.16080	328	3022
13	26.7511	3.32984	13	0.14980	335	2995
14	27.6414	3.22457	11	0.17420	272	2656
15	28.8062	3.09678	27	0.16880	688	6568
16	29.4200	3.03355	12	0.18700	315	3812
17	29.6916	3.00642	65	0.17360	1647	14121
18	29.9843	2.97773	17	0.18330	439	4665
19	30.4169	2.93636	4	0.16390	93	917
20	31.0784	2.87535	3	0.15680	87	882
21	32.2948	2.76977	26	0.16090	651	6016
22	34.1649	2.62232	19	0.24330	491	6587
23	35.0400	2.55881	3	0.16660	84	1159
24	35.2000	2.54754	5	0.23140	124	1328
25	36.2228	2.47792	3	0.22960	86	1220
26	37.8751	2.37353	4	0.22680	96	1184
27	39.1998	2.29632	8	0.24830	207	3189
28	40.8791	2.20578	5	0.16480	139	1711
29	41.8962	2.15455	4	0.21640	102	1131
30	42.3800	2.13107	4	0.17720	93	1161
31	42.8537	2.10860	8	0.27740	196	2950
32	43.2513	2.09014	3	0.22840	82	965
33	44.1000	2.05186	5	0.20280	117	2531
34	44.4000	2.03869	6	0.00000	152	0
35	44.6000	2.03001	5	0.00000	124	0
36	44.8398	2.01971	7	0.19340	179	2303
37	45.2368	2.00290	5	0.15160	116	967

3. 5-Azacytidine Hemisulfate Salt Methanol Solvate

[00309] ^{1}H NMR (DMSO-d_6) δ 8.76 (s, 1H), 8.69 (s, 1H), 8.25 (s, 1H), 5.61 (d, 1H, J = 2.4 Hz), 4.09 (m, 1H), 4.02 (m, 1H), 3.87 (m, 1H), 3.72 (m, 1H), 3.57 (m, 1H), 3.14 (s, 3H) (CH_3OH).

[00310] ^{13}C NMR (DMSO-d_6) δ 162.8, 157.5, 150.2, 90.2, 84.5, 74.2, 68.5, 59.7, 48.9 (CH_3OH).

[00311] LC-MS ESI: m/z 245.2 ($M + H^+$).

[00312] The IR spectrum of 5-azacytidine hemisulfate salt methanol solvate is shown in **Figure 11**; the DSC plot of 5-azacytidine hemisulfate salt methanol solvate is shown in

Figure 12. The XRPD pattern of 5-azacytidine hemisulfate salt methanol solvate is shown in **Figure 13**. The XRPD data is further provided in the table below:

Table 27: XRPD – Strongest 3 peaks

no.	Peak no.	2Theta (deg)	d (A)	I/I1	FWHM (deg)	Intensity (Counts)	Integrated Int (Counts)
1	15	20.7054	4.28642	100	0.24550	1073	14977
2	7	15.5303	5.70116	95	0.27540	1014	15758
3	17	22.4492	3.95725	87	0.25300	937	13242

Table 28: XRPD – Peak Data List

Peak no.	2Theta (deg)	d (A)	I/I1	FWHM (deg)	Intensity (Counts)	Integrated Int (Counts)
1	7.7800	11.35446	3	0.18660	35	484
2	8.0200	11.01521	7	0.20000	70	752
3	9.2841	9.51805	10	0.32170	111	2488
4	10.2184	8.64979	7	0.27690	75	1498
5	13.8860	6.37234	6	0.33200	61	1289
6	14.7439	6.00342	28	0.30600	299	5182
7	15.5303	5.70116	95	0.27540	1014	15758
8	16.3058	5.43172	33	0.33420	351	6494
9	16.8200	5.26680	10	0.26340	105	1672
10	17.5682	5.04415	9	0.22980	93	1144
11	18.6876	4.74445	17	0.31930	183	3260
12	19.3369	4.58658	17	0.24440	179	2462
13	19.9800	4.44038	33	0.30820	350	6176
14	20.2200	4.38821	19	0.00000	203	0
15	20.7054	4.28642	100	0.24550	1073	14977
16	21.0800	4.21109	9	0.42660	99	3355
17	22.4492	3.95725	87	0.25300	937	13242
18	23.0800	3.85050	7	0.19000	75	834
19	23.3000	3.81464	12	0.26880	130	1613
20	24.0475	3.69773	9	0.27910	100	1609
21	24.4200	3.64216	7	0.16000	77	869
22	24.7239	3.59808	42	0.26190	448	5628
23	25.1600	3.53669	23	0.34900	250	4362
24	25.5017	3.49007	51	0.23860	542	6193
25	26.2202	3.39604	16	0.23670	176	2431
26	27.0600	3.29252	4	0.17000	45	730
27	27.4793	3.24323	26	0.35370	284	6993
28	28.0400	3.17963	14	0.00000	151	0
29	28.4200	3.13798	17	0.00000	180	0
30	29.0313	3.07328	19	0.29870	208	6146
31	29.7535	3.00030	5	0.40710	59	1075
32	30.1250	2.96415	5	0.33000	57	1037
33	31.6000	2.82907	10	0.28000	107	2309
34	31.9600	2.79802	8	0.00000	82	0
35	32.1200	2.78445	8	0.47560	83	1624
36	32.9980	2.71234	7	0.31600	72	1298
37	34.1600	2.62268	6	0.35200	65	1547
38	34.4200	2.60347	6	0.00000	60	0
39	34.8400	2.57304	7	0.32000	70	1862
40	35.4883	2.52750	12	0.32790	126	2292
41	36.6527	2.44984	7	0.23680	78	886
42	37.0600	2.42384	5	0.37200	50	855

Peak no.	2Theta (deg)	d (A)	I/I1	FWHM (deg)	Intensity (Counts)	Integrated Int (Counts)
43	37.3600	2.40507	3	0.24500	37	481
44	38.2516	2.35103	7	0.29670	70	1353
45	39.6800	2.26963	4	0.16800	40	453
46	39.9326	2.25585	8	0.45730	85	1544
47	40.8125	2.20923	5	0.33500	53	867

4. 5-Azacytidine Mesylate Salt Methanol Solvate

[00313] ^{1}H NMR (DMSO-d_6) δ 9.74 (s, 1H), 9.07 (s, 1H), 8.94 (s, 1H), 5.58 (d, 1H, J = 0.9 Hz), 4.11 (m, 1H), 4.05 (m, 1H), 3.90(m, 1H), 3.78 (m, 1H), 3.59 (m, 1H), 3.15 (s, 3H) (CH_3OH), 2.49 (s, 3H) (CH_3SO_3H).

[00314] ^{13}C NMR (DMSO-d_6) δ 160.0, 158.3, 146.9, 90.7, 84.4, 74.2, 67.9, 59.2, 48.9 (CH_3OH), 40.0 (CH_3SO_3H).

[00315] LC-MS ESI: m/z 245.2 ($M + H^+$).

[00316] The IR spectrum of 5-azacytidine mesylate salt methanol solvate is shown in **Figure 14**; the DSC plot of 5-azacytidine mesylate salt methanol solvate is shown in **Figure 15**. The XRPD pattern of 5-azacytidine mesylate salt methanol solvate is shown in **Figure 16**. The XRPD data is further provided in the table below:

Table 29: XRPD – Strongest 3 peaks

no.	Peak no.	2Theta (deg)	d (A)	I/I1	FWHM (deg)	Intensity (Counts)	Integrated Int (Counts)
1	14	18.4114	4.81500	100	0.71380	796	27178
2	24	27.8449	3.20147	66	0.40900	527	11554
3	5	9.0287	9.78671	38	0.43740	299	7573

Table 30: XRPD – Peak Data List

Peak no.	2Theta (deg)	d (A)	I/I1	FWHM (deg)	Intensity (Counts)	Integrated Int (Counts)
1	5.1200	17.24595	3	0.36000	24	1341
2	5.8400	15.12125	4	0.00000	31	0
3	6.4800	13.62916	5	0.00000	36	0
4	7.3400	12.03410	10	0.74660	81	4815
5	9.0287	9.78671	38	0.43740	299	7573
6	9.7322	9.08079	19	0.50890	152	4481
7	11.6904	7.56374	11	0.39420	87	1951
8	13.1008	6.75245	11	0.37830	91	1818
9	13.8600	6.38423	6	0.32800	48	1041
10	14.7266	6.01044	17	0.62670	134	4254
11	15.5183	5.70554	16	0.46160	131	3257
12	16.6612	5.31664	20	0.49250	156	4279
13	17.5600	5.04649	14	0.76000	112	4315
14	18.4114	4.81500	100	0.71380	796	27178
15	19.5860	4.52880	20	0.50300	160	4059
16	20.2466	4.38250	24	0.50670	190	4721

Peak no.	2Theta (deg)	d (A)	I/I1	FWHM (deg)	Intensity (Counts)	Integrated Int (Counts)
17	21.3800	4.15267	15	1.12000	122	6951
18	22.0200	4.03340	19	0.00000	149	0
19	23.0149	3.86124	35	0.79870	280	12958
20	23.5000	3.78262	18	0.00000	143	0
21	24.5000	3.63045	15	0.42000	122	6939
22	25.8160	3.44829	5	0.51200	39	1204
23	26.3750	3.37646	5	0.45000	36	649
24	27.8449	3.20147	66	0.40900	527	11664
25	28.8800	3.08903	10	0.45000	80	3453
26	29.7000	3.00559	3	0.24000	26	377
27	30.1800	2.95887	5	0.46000	36	929
28	31.4515	2.84209	5	0.47300	42	1195
29	32.4275	2.75874	10	0.57500	78	2146
30	33.4000	2.68060	4	0.56000	29	1319
31	37.2825	2.40989	7	0.41500	55	1282
32	37.8893	2.37268	3	0.24530	27	410

5. 5-Azacytidine Mono-Hydrobromide Salt

[00317] ^{1}H NMR (DMSO-d_6) δ 9.50 (s, 1H), 8.91 (s, 1H), 8.56 (s, 1H), 5.60 (d, 1H, J = 1.7 Hz), 4.12 (m, 1H), 4.05 (m, 1H), 3.90(m, 1H), 3.77 (m, 1H), 3.59 (m, 1H).

[00318] ^{13}C NMR (DMSO-d_6) δ 160.0, 158.3, 147.0, 90.7, 84.6, 74.2, 68.1, 59.3.

[00319] LC-MS ESI: m/z 245.0 ($M + H^+$).

[00320] The IR spectrum of 5-azacytidine hydrobromide salt is shown in **Figure 17**; the DSC plot of 5-azacytidine hydrobromide salt is shown in **Figure 18**. The XRPD pattern of 5-azacytidine hydrobromide salt is shown in **Figure 19**. The XRPD data is further provided in the table below:

Table 31: XRPD – Strongest 3 peaks

no.	Peak no.	2Theta (deg)	d (A)	I/I1	FWHM (deg)	Intensity (Counts)	Integrated Int (Counts)
1	17	29.3754	3.03806	100	0.21090	3561	45176
2	7	22.3855	3.96836	99	0.23060	3520	53273
3	16	28.9891	3.07766	54	0.27900	1931	34036

Table 32: XRPD – Peak Data List

Peak no.	2Theta (deg)	d (A)	I/I1	FWHM (deg)	Intensity (Counts)	Integrated Int (Counts)
1	13.7653	6.42794	5	0.30940	163	3756
2	16.9160	5.23713	38	0.30120	1369	24687
3	17.2377	5.14011	52	0.25120	1867	26905
4	18.6943	4.74276	17	0.25010	623	10585
5	20.0218	4.43120	37	0.26200	1322	22877
6	21.1485	4.19760	41	0.23030	1468	21781
7	22.3855	3.96836	99	0.23060	3520	53273
8	23.6281	3.76240	33	0.22880	1160	17423
9	24.2286	3.67050	6	0.20170	212	2901

Peak no.	2Theta (deg)	d (A)	I/I1	FWHM (deg)	Intensity (Counts)	Integrated Int (Counts)
10	24.7088	3.60024	29	0.22290	1040	14307
11	25.7251	3.46027	29	0.20130	1021	13071
12	26.1470	3.40538	5	0.17260	192	3293
13	26.4765	3.36374	26	0.21120	910	11758
14	27.3328	3.26028	23	0.22590	811	11753
15	27.7680	3.21016	10	0.20890	359	4594
16	28.9891	3.07766	54	0.27900	1931	34036
17	29.3754	3.03806	100	0.21090	3561	45176
18	29.7689	2.99879	22	0.21720	776	10904
19	30.3935	2.93857	12	0.21880	443	6695
20	31.8694	2.80577	39	0.22910	1391	20971
21	33.2273	2.69414	20	0.20570	712	9758
22	33.7458	2.65392	31	0.20350	1096	14388
23	34.1908	2.62039	12	0.19960	412	5206
24	34.8996	2.56878	9	0.21060	305	4108
25	36.6147	2.45229	11	0.21930	408	6358
26	37.3233	2.40735	6	0.20880	218	3006
27	37.9283	2.37033	14	0.28880	498	8915
28	38.8907	2.31386	10	0.32320	370	6970
29	39.4410	2.28283	4	0.14740	130	1135
30	39.6960	2.26875	3	0.32120	119	2296
31	40.1470	2.24430	8	0.18330	273	3009
32	40.6990	2.21512	7	0.41120	252	4839
33	40.9030	2.20455	4	0.00000	148	0
34	41.1653	2.19110	6	0.22630	208	3523
35	41.7244	2.16303	7	0.17980	258	2908
36	42.2041	2.13954	12	0.22730	444	6246
37	43.2388	2.09071	14	0.45860	516	13010
38	43.6740	2.07088	9	0.26780	322	5023
39	44.1942	2.04770	12	0.30470	412	7454
40	44.6363	2.02844	15	0.22970	518	7908
41	45.9267	1.97441	4	0.15080	153	1806
42	47.6170	1.90819	4	0.21110	144	1915
43	47.9580	1.89541	3	0.24780	120	2775
44	48.2300	1.88536	4	0.00000	144	0
45	48.3830	1.87975	3	0.36980	112	2169
46	48.7606	1.86608	3	0.24530	109	1694

6. 2',3',5'-Triacetyl-5-Azacytidine

[00321] ^{1}H NMR (DMSO-d_6) δ 8.32 (s, 1H), 7.72 (d, 2H, J = 9.1 Hz), 5.66 (d, 1H, J = 3.2 Hz), 5.53 (m, 1H), 5.41 (t, 1H, J = 6.6 Hz), 4.32 (dd, 1H, JJ = 2.8, 11.6 Hz), 4.20 (m, 1H), 4.14 (m, 1H), 2.04 (s, 1H), 2.02 (s, 1H), 2.00 (s, 1H).

[00322] ^{13}C NMR (DMSO-d_6) δ 170.5, 169.9, 169.7, 166.2, 158.0, 152.9, 90.8, 79.3, 72.9, 70.0, 63.2, 20.8, 20.6, 20.6.

[00323] LC-MS ESI: m/z 371.2 ($M + H^+$), 393.1 ($M + Na^+$).

[00324] The IR spectrum of 2',3',5'-triacetyl-5-azacytidine is shown in **Figure 20**.

[00325] The embodiments described above are intended to be merely exemplary, and those skilled in the art will recognize, or will be able to ascertain using no more than routine experimentation, numerous equivalents of specific compounds, materials, and procedures. All such equivalents are considered to be within the scope of the disclosure.

[00326] All of the patents, patent applications and publications referred to herein are incorporated herein in their entireties. Citation or identification of any reference in this application is not an admission that such reference is available as prior art to this application. The full scope of the disclosure is better understood with reference to the appended claims.

WHAT IS CLAIMED:

1. A process for preparing 5-azacytidine, or a salt, solvate, hydrate, or polymorph thereof, comprising the steps of reacting a silylated 5-azacytosine with a protected β-D-ribofuranose in the presence of a metallic Lewis acid to yield a protected 5-azacytidine, deprotecting the protected 5-azacytidine to yield 5-azacytidine, and purifying the 5-azacytidine to yield 5-azacytidine, or a salt, solvate, hydrate, or polymorph thereof, that is substantially free of metal-based impurities.

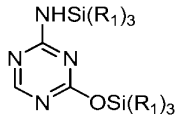
2. The process of claim 1, wherein the total metal-based impurities in the purified 5-azacytidine, or a salt, solvate, hydrate, or polymorph thereof, are less than about 20 ppm w/w.

3. The process of claim 1, comprising the steps of:
(a) reacting 5-azacytosine with a silylating reagent to yield a silylated 5-azacytosine;
(b) reacting the silylated 5-azacytosine with an acyl protected β-D-ribofuranose in the presence of a metallic Lewis acid; and quenching the reaction with water and at least one neutralizing reagent to yield a protected 5-azacytidine;
(c) reacting the protected 5-azacytidine with a base, selected from the group consisting of alkoxide, ammonia, and tetra-substituted ammonium hydroxide, in an alcohol to yield 5-azacytidine; and
(d) contacting the 5-azacytidine from step (c) with an acid in an organic solvent to yield a salt of 5-azacytidine.

4. The process of claim 3, further comprising the step of:
(e) contacting the salt of 5-azacytidine from step (d) with a base in an organic solvent to yield 5-azacytidine free base.

5. The process of claim 4, further comprising the step of:
(f) re-crystallizing the 5-azacytidine from step (e).

6. The process of any one of claims 3 to 5, wherein the silylated 5-azacytosine is a compound of formula (A):



(A),

wherein each R_1 is independently optionally substituted C_1-C_{10} alkyl, optionally substituted C_3-C_{10} cycloalkyl, or optionally substituted C_6-C_{10} aryl.

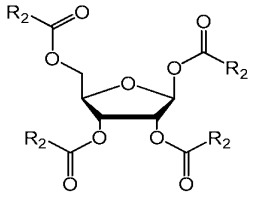
7. The process of claim 6, wherein R_1 is methyl.

8. The process of any one of claims 3 to 7, wherein the silylating reagent used in step (a) is a trimethylsilyl reagent.

9. The process of any one of claims 3 to 8, wherein the silylating reagent used in step (a) is hexamethyldisilazane.

10. The process of any one of claims 3 to 9, wherein the silylation reaction of step (a) is carried out in the presence of ammonium sulfate.

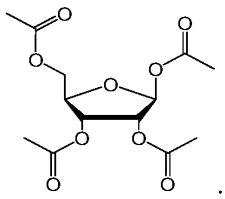
11. The process of any one of claims 3 to 10, wherein the protected β-D-ribofuranose is a compound of formula (B):



(B),

wherein each R_2 is independently hydrogen, optionally substituted C_1-C_{10} alkyl, optionally substituted C_3-C_{10} cycloalkyl, or optionally substituted C_6-C_{10} aryl.

12. The process of any one of claims 3 to 10, wherein the protected β-D-ribofuranose is:



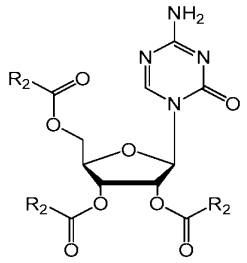
.

13. The process of any one of claims 3 to 12, wherein the metallic Lewis acid is stannic chloride or ferric chloride.

14. The process of any one of claims 3 to 12, wherein the metallic Lewis acid is stannic chloride.

15. The process of any one of claims 3 to 12, wherein the metallic Lewis acid is ferric chloride.

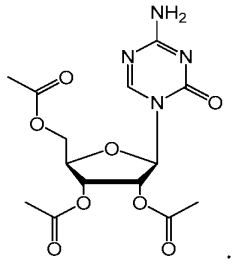
16. The process of any one of claims 3 to 11 and 13 to 15, wherein the protected 5-azacytidine is a compound of formula (C):



(C),

wherein each R_2 is independently hydrogen, optionally substituted C_1-C_{10} alkyl, optionally substituted C_3-C_{10} cycloalkyl, or optionally substituted C_6-C_{10} aryl.

17. The process of any one of claims 3 to 15, wherein the protected 5-azacytidine is:



.

18. The process of any one of claims 3 to 17, wherein the coupling reaction of step (b) is carried out in a solvent with low water solubility.

19. The process of claim 18, wherein the solvent with low water solubility is dichloromethane.

20. The process of any one of claims 3 to 19, wherein the coupling reaction of step (b) is carried out at a temperature of between about 0°C and about 5°C.

21. The process of any one of claims 3 to 20, wherein the neutralizing reagent in step (b) is a carbonate or bicarbonate salt.

22. The process of any one of claims 3 to 20, wherein the neutralizing reagent in step (b) is sodium bicarbonate.

23. The process of any one of claims 3 to 22, wherein the reaction of step (b) is quenched at a temperature of less than about 10°C.

24. The process of any one of claims 3 to 23, wherein the base in step (c) is an alkoxide.

25. The process of claim 24, wherein the alkoxide is sodium methoxide.

26. The process of any one of claims 3 to 25, wherein the alcohol in step (c) is methanol.

27. The process of any one of claims 3 to 26, wherein the acid used in step (d) is hydrochloric acid, hydrobromic acid, sulfuric acid, or methanesulfonic acid.

28. The process of any one of claims 3 to 26, wherein the acid used in step (d) is hydrochloric acid.

29. The process of any one of claims 3 to 28, wherein the organic solvent used in step (d) is an alcohol.

30. The process of any one of claims 3 to 29, wherein the organic solvent used in step (d) is methanol.

31. The process of any one of claims 3 to 30, wherein the salt of 5-azacytidine formed in step (d) is 5-azacytidine mono-hydrochloride salt.

32. The process of any one of claims 4 to 31, wherein the base used in step (e) is an organic base.

33. The process of claim 32, wherein the organic base is triethylamine.

34. The process of any one of claims 4 to 33, wherein the organic solvent used in step (e) is an alcohol.

35. The process of any one of claims 4 to 34, wherein the organic solvent used in step (e) is methanol.

36. The process of any one of claims 5 to 35, wherein step (f) comprises the steps of:
(1) dissolving 5-azacytidine from step (e) in dimethylsulfoxide at a temperature sufficient to allow the 5-azacytidine to dissolve; and optionally filtering the solution to remove insoluble particles;
(2) adding an anti-solvent to the solution of step (1); and
(3) cooling the mixture of step (2) wherein 5-azacytidine re-crystallizes.

37. The process of claim 36, wherein the anti-solvent of step (f)(2) is an alcohol.

38. The process of claim 36 or 37, wherein the anti-solvent of step (f)(2) is methanol.

39. The process of any one of claims 36 to 38, wherein step (f) further comprises the steps of:
(4) collecting the re-crystallized 5-azacytidine from step (3) by filtration; and
(5) drying the 5-azacytidine from step (4) under vacuum.

40. A salt of 5-azacytidine, selected from the group consisting of hydrochloride salt, hydrobromide salt, and sulfate salt.

41. The salt of claim 40, wherein the salt is a mono-hydrochloride salt of 5-azacytidine.

42. The salt of claim 40, wherein the salt is a mono-hydrobromide salt of 5-azacytidine.

43. The salt of claim 40, wherein the salt is a hemisulfate salt of 5-azacytidine, which is a methanol solvate.

44. The salt of claim 40, wherein the salt is a mesylate salt of 5-azacytidine.

45. The salt of any one of claims 40 to 44, wherein the salt is substantially free of impurities.

46. The salt of any one of claims 40 to 45, wherein the salt is substantially crystalline.

47. 5-Azacytidine, or a salt, solvate, hydrate, or polymorph thereof, prepared by the process of any one of claims 1 to 39.

48. 5-Azacytidine, or a salt, solvate, hydrate, or polymorph thereof, obtainable from the process of any one of claims 1 to 39.

49. The 5-azacytidine, or a salt, solvate, or hydrate thereof, which is substantially crystalline.

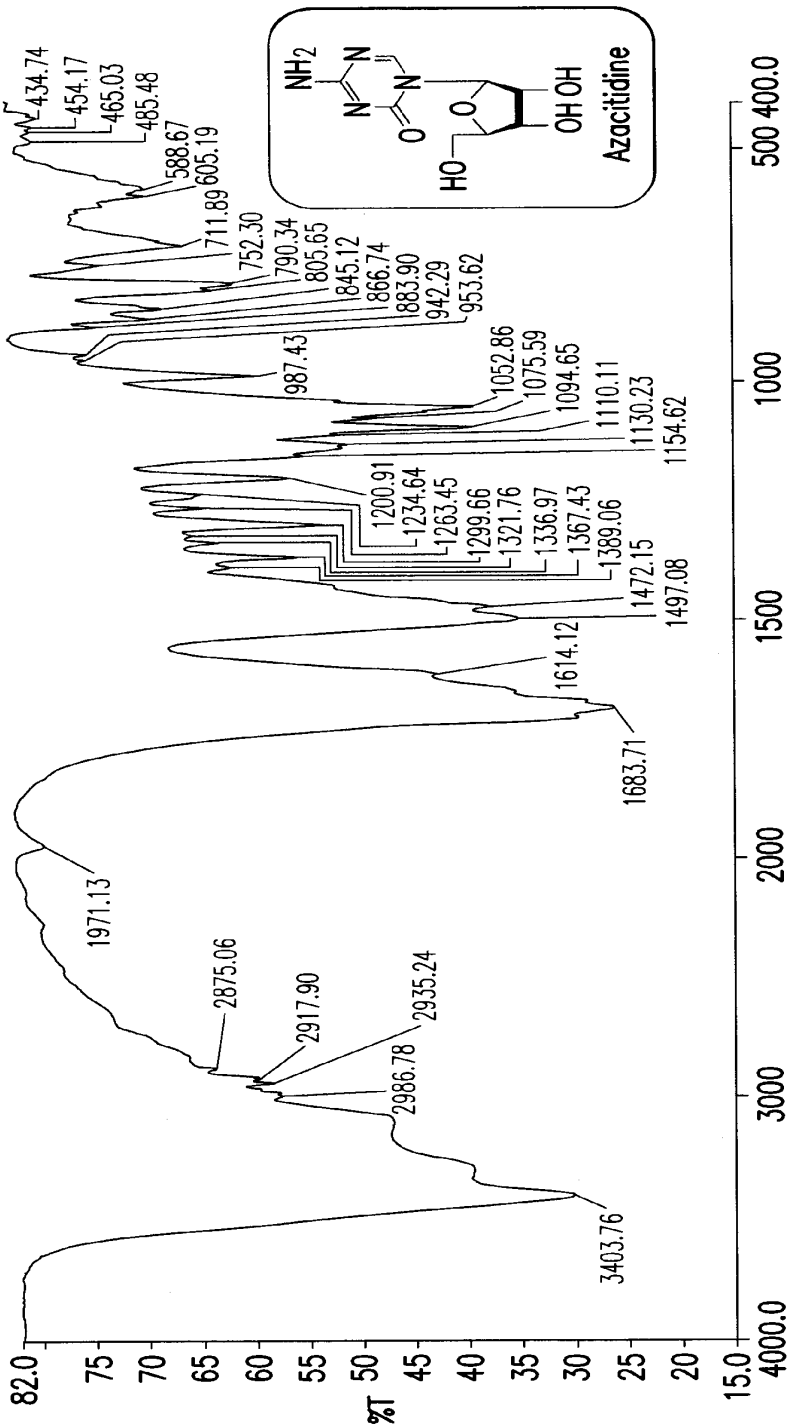


FIG. 1

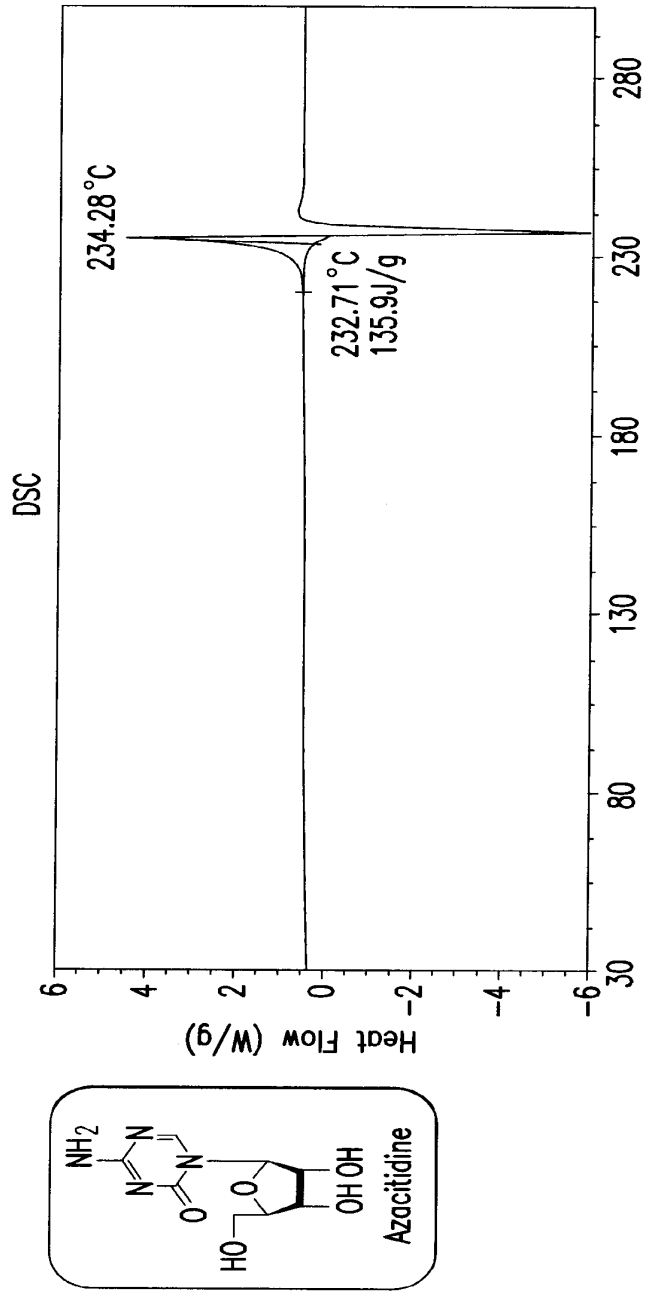

DSC
234.28°C
232.71°C
135.9J/g
Heat Flow (W/g)
Temperature (°C)
6
4
2
0
-2
-4
-6
30
80
130
180
230
280
NH2
N
N
N
N
O
O
HO
OH OH
Azacitidine

FIG.2

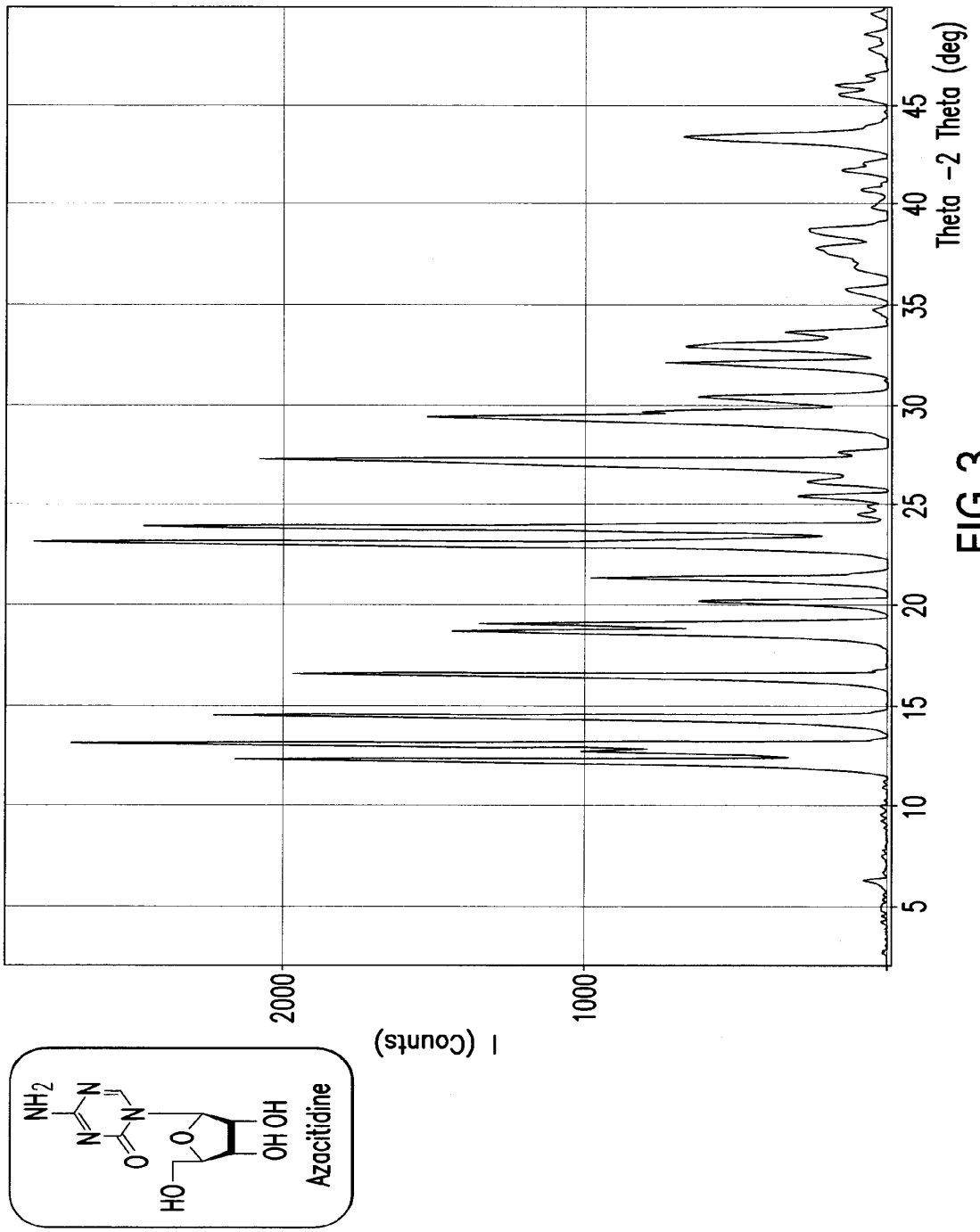

I (Counts)
2000
1000
5
10
15
20
25
30
35
40
45
Theta -2 Theta (deg)
NH2
N
N
N
O
O
OH OH
HO
Azacitidine

FIG.3

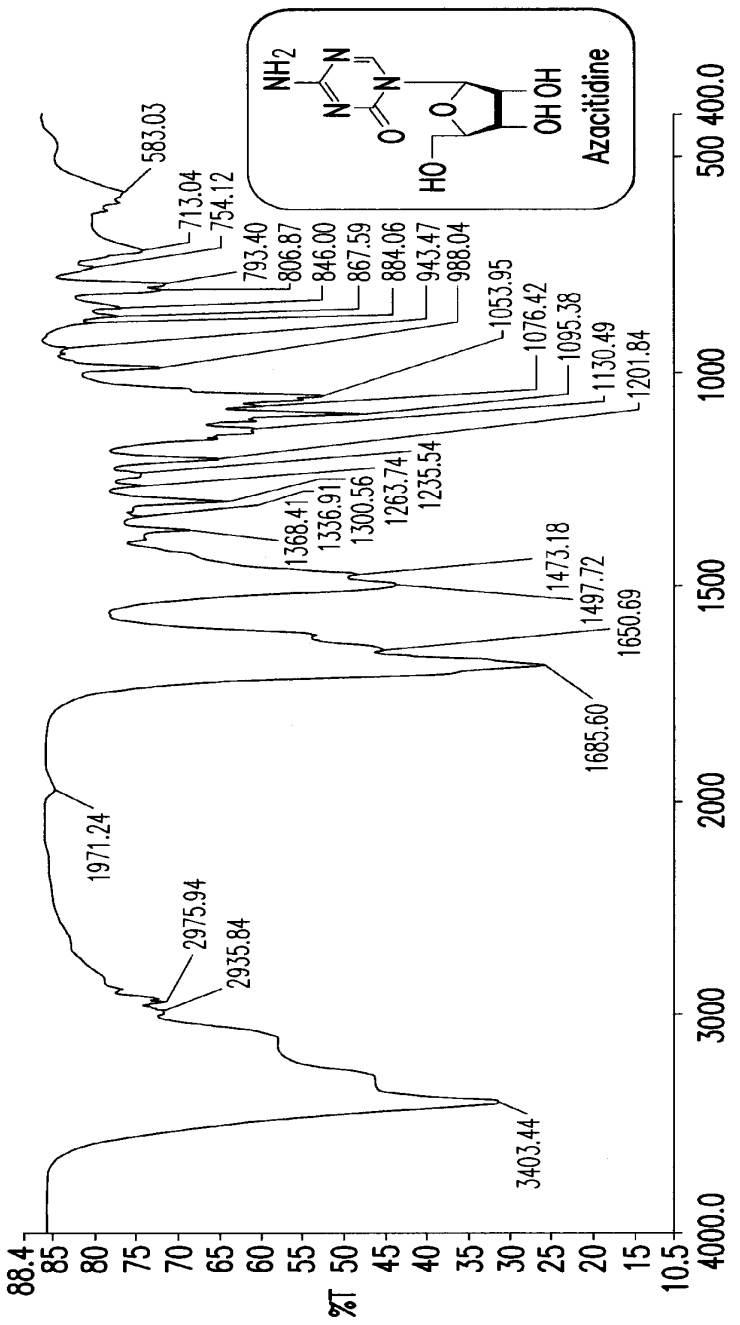

583.03
713.04
754.12
793.40
806.87
846.00
867.59
884.06
943.47
988.04
1053.95
1076.42
1095.38
1130.49
1201.84
1235.54
1263.74
1300.56
1336.91
1368.41
1473.18
1497.72
1650.69
1685.60
1971.24
2975.94
2935.84
3403.44
NH2
HO
OH OH
Azacitidine
%T
cm-1
88.4
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10.5
4000.0
3000
2000
1500
1000
500 400.0

FIG.4

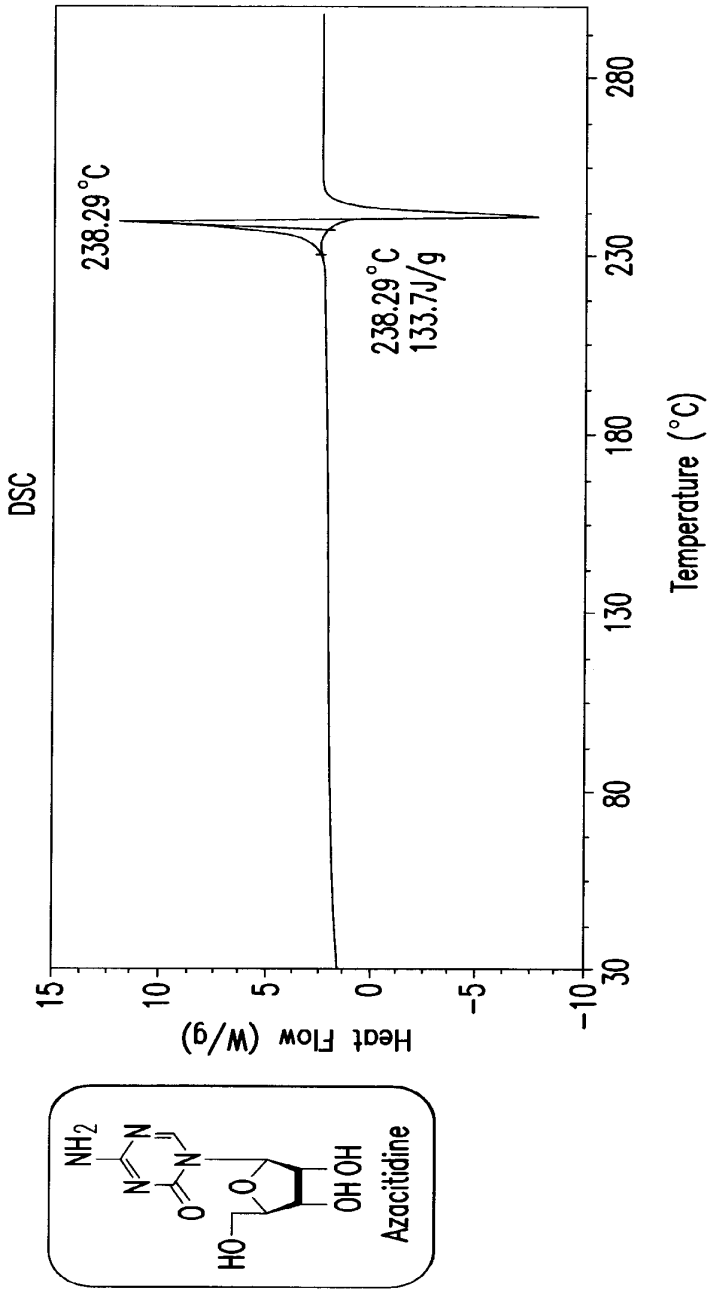

DSC
238.29°C
238.29°C
133.7J/g
Heat Flow (W/g)
15
10
5
0
-5
-10
30
80
130
180
230
280
Temperature (°C)
NH2
N
N
N
O
O
HO
OH OH
Azacitidine

FIG.5

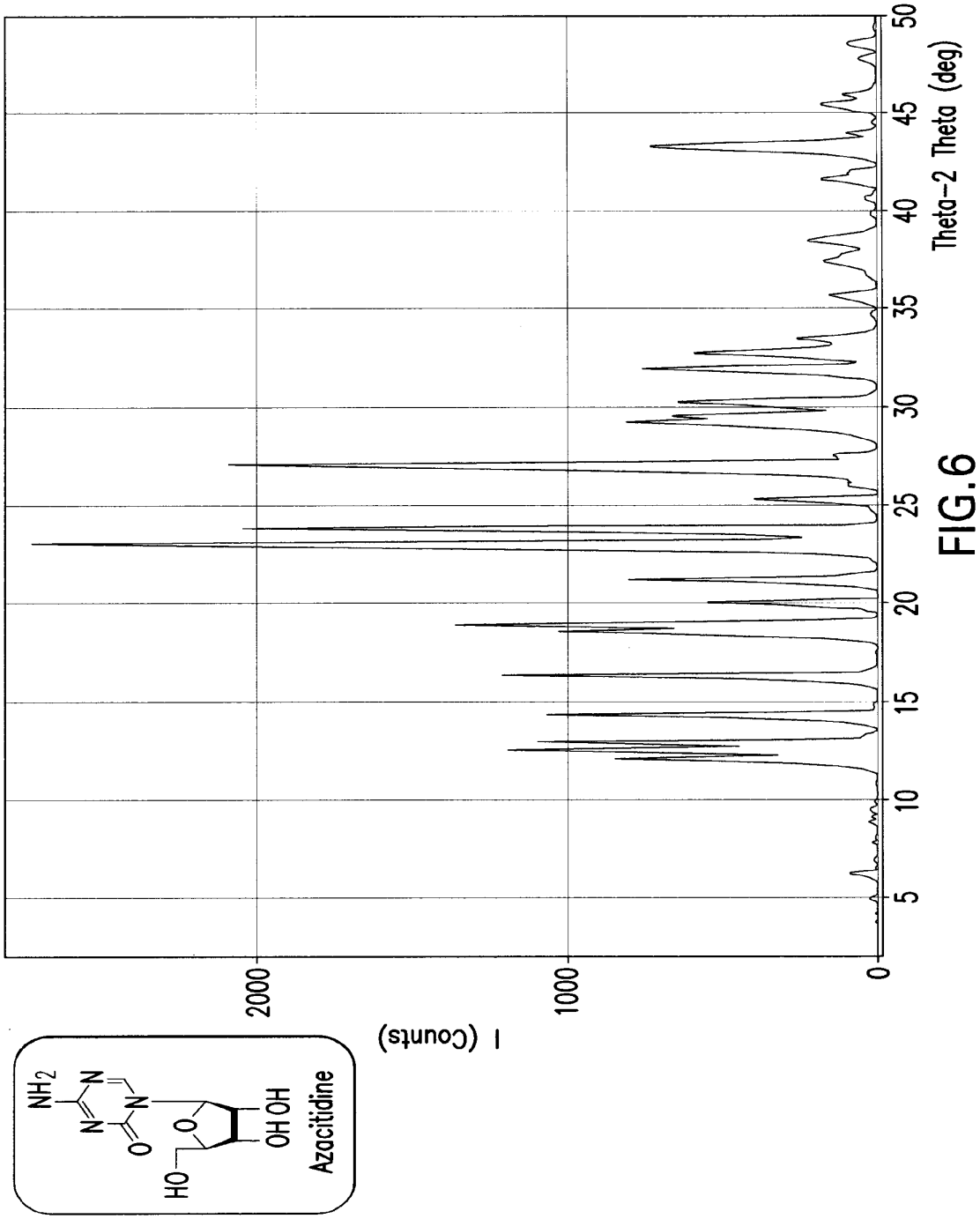

I (Counts)
2000
1000
0
5
10
15
20
25
30
35
40
45
50
Theta-2 Theta (deg)
NH2
N
N
N
O
O
OH OH
HO
Azacitidine

FIG.6

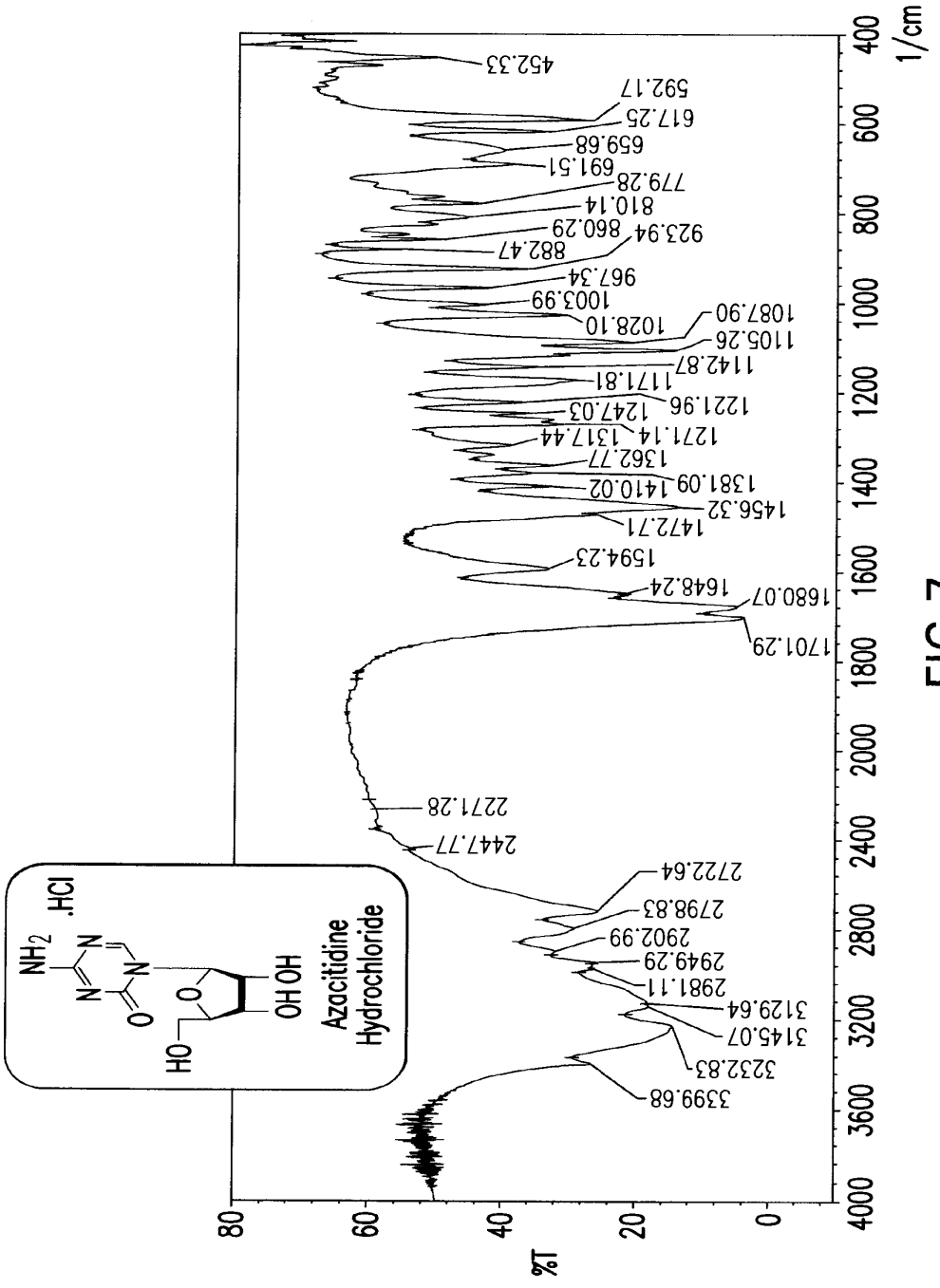

452.33
592.17
617.25
659.68
691.51
779.28
810.14
860.29
882.47
923.94
967.34
1003.99
1028.10
1087.90
1105.26
1142.87
1171.81
1221.96
1247.03
1271.14
1317.44
1362.77
1381.09
1410.02
1456.32
1472.71
1594.23
1648.24
1680.07
1701.29
2271.28
2447.77
2722.64
2798.83
2902.99
2949.29
2981.11
3129.64
3145.07
3232.83
3399.68
Azacitidine Hydrochloride
%T
1/cm

FIG.7

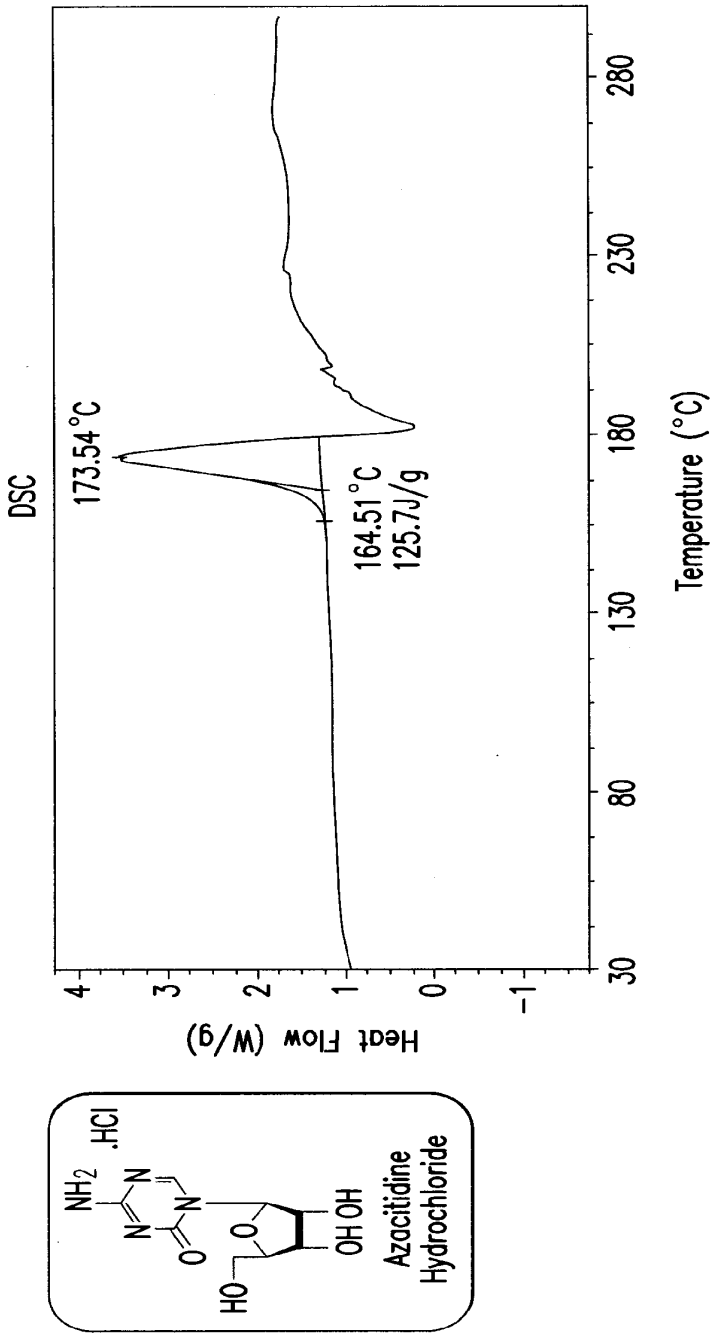

DSC
173.54°C
164.51°C
125.7J/g
Heat Flow (W/g)
Temperature (°C)
Azacitidine
Hydrochloride

FIG.8

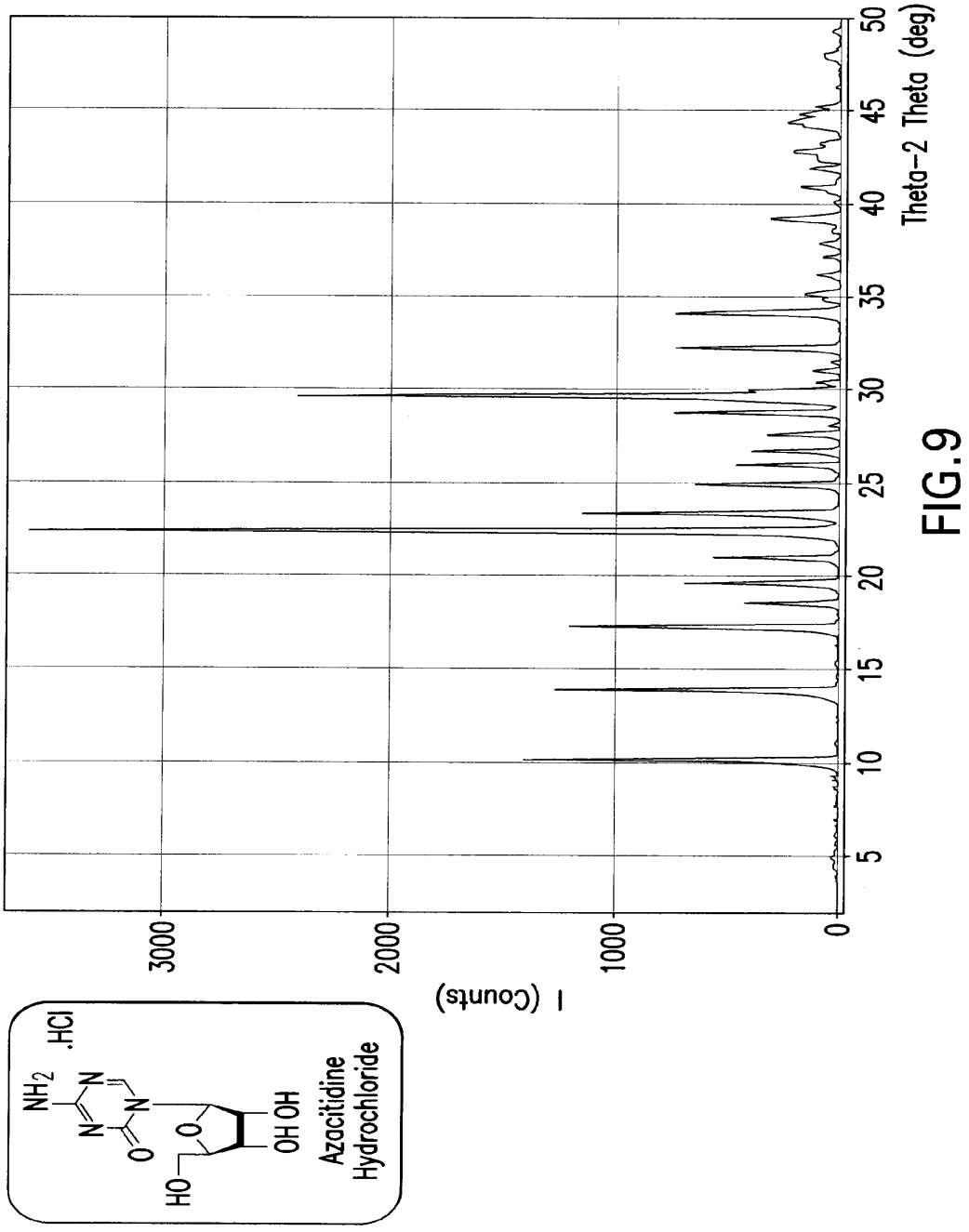

I (Counts)
3000
2000
1000
0
5
10
15
20
25
30
35
40
45
50
Theta-2 Theta (deg)
.HCl
NH2
N
N
O
O
OH OH
HO
Azacitidine
Hydrochloride

FIG.9

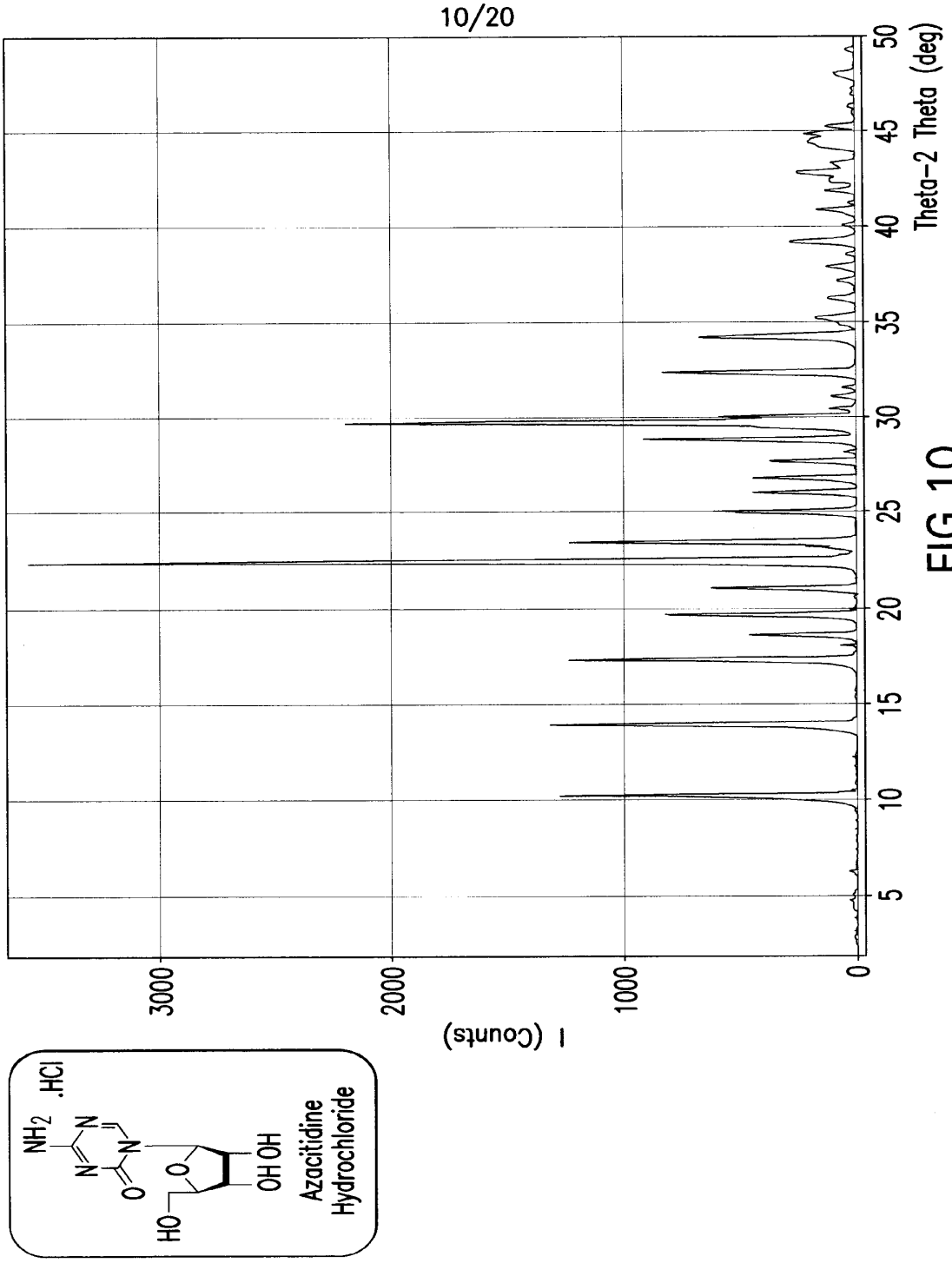

I (Counts)
3000
2000
1000
0
5
10
15
20
25
30
35
40
45
50
Theta-2 Theta (deg)
NH2 .HCl
N
N
N
N
O
O
HO
OH OH
Azacitidine
Hydrochloride

FIG.10

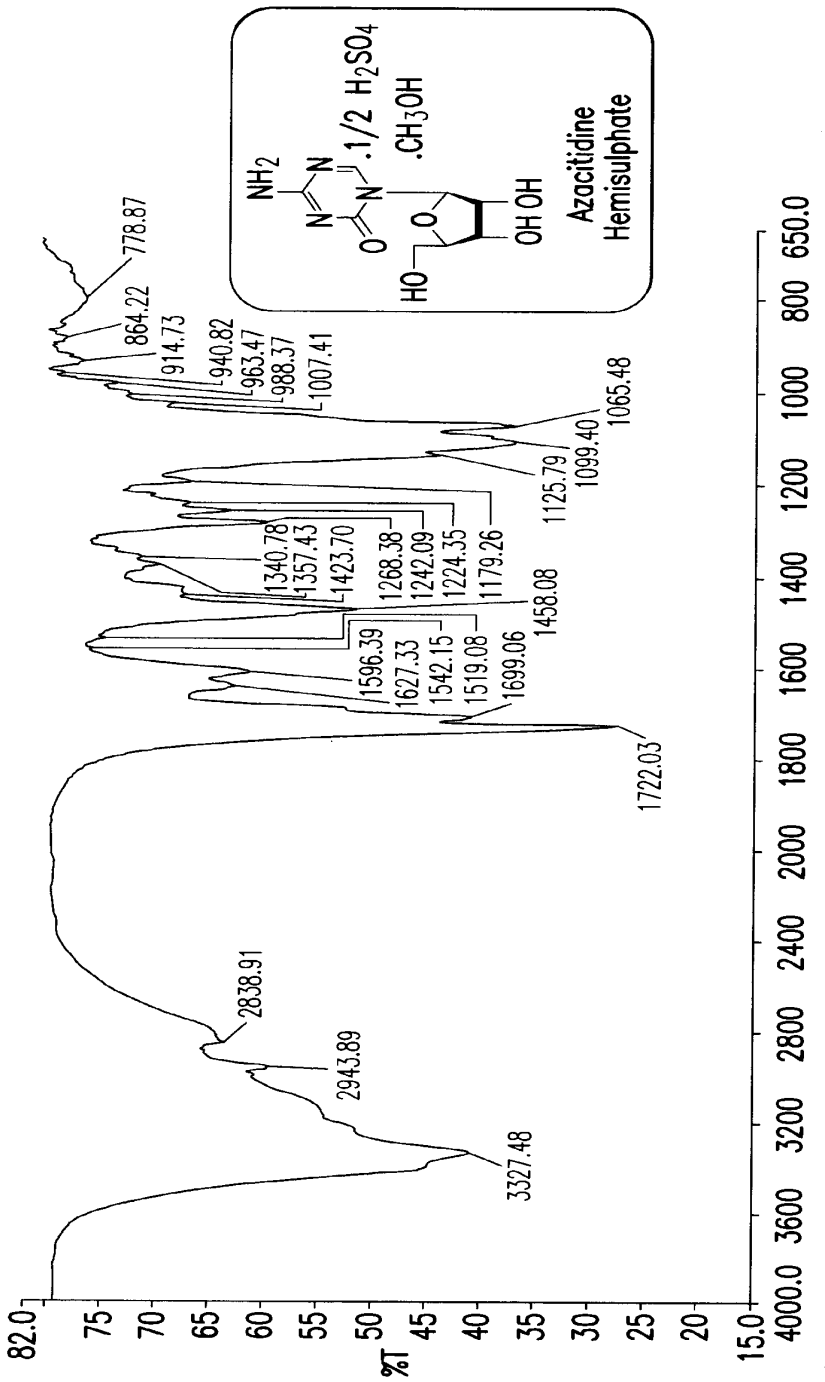

Azacitidine Hemisulphate
NH2
.1/2 H2SO4
.CH3OH
HO
OH OH
%T
cm-1
82.0
75
70
65
60
55
50
45
40
35
30
25
20
15.0
4000.0
3600
3200
2800
2400
2000
1800
1600
1400
1200
1000
800
650.0
778.87
864.22
914.73
940.82
963.47
988.37
1007.41
1065.48
1099.40
1125.79
1179.26
1224.35
1242.09
1268.38
1340.78
1357.43
1423.70
1458.08
1519.08
1542.15
1596.39
1627.33
1699.06
1722.03
2838.91
2943.89
3327.48

FIG. 11

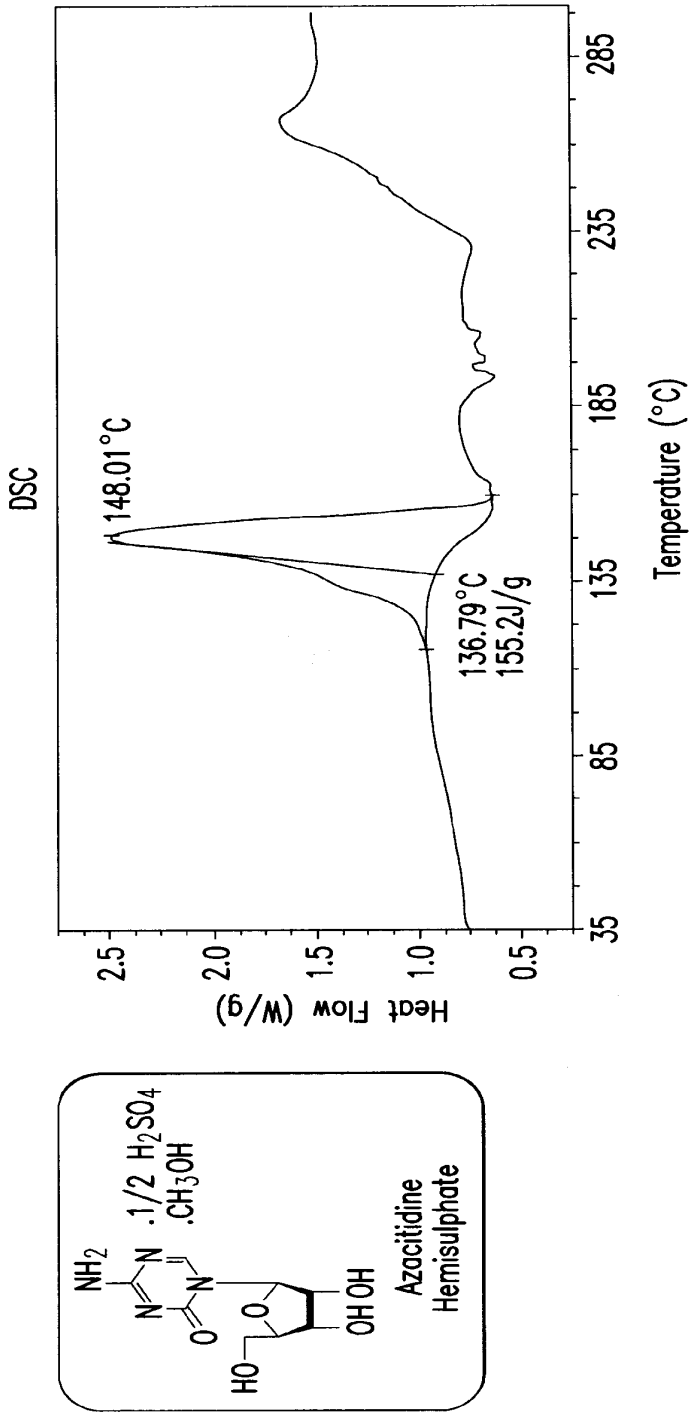

DSC
148.01°C
136.79°C
155.2J/g
Heat Flow (W/g)
Temperature (°C)
2.5
2.0
1.5
1.0
0.5
35
85
135
185
235
285
NH2
.1/2 H2SO4
.CH3OH
HO
OH OH
Azacitidine
Hemisulphate

FIG.12

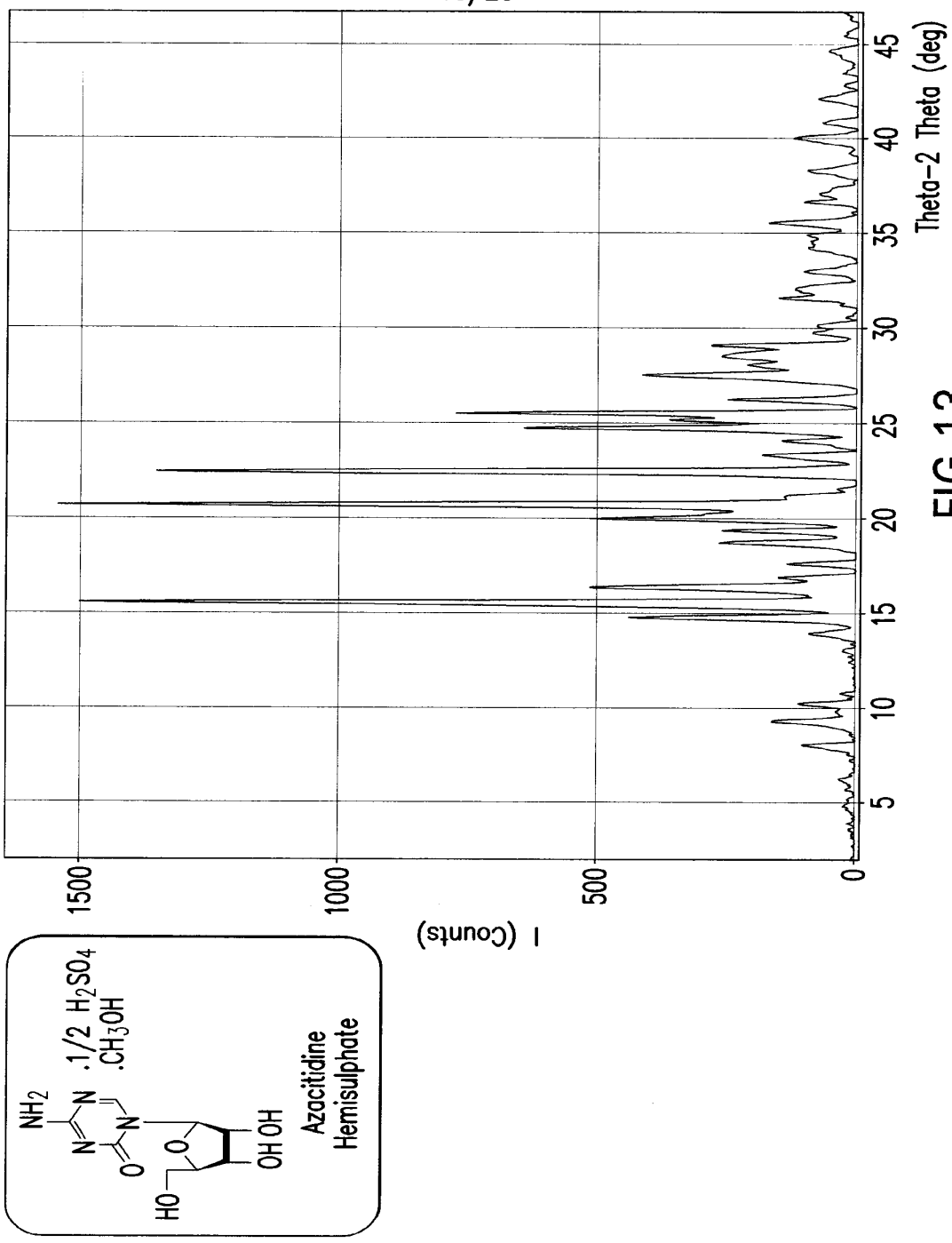

I (Counts)
1500
1000
500
0
5
10
15
20
25
30
35
40
45
Theta-2 Theta (deg)
NH2
.1/2 H2SO4
.CH3OH
HO
OH OH
Azacitidine
Hemisulphate

FIG.13

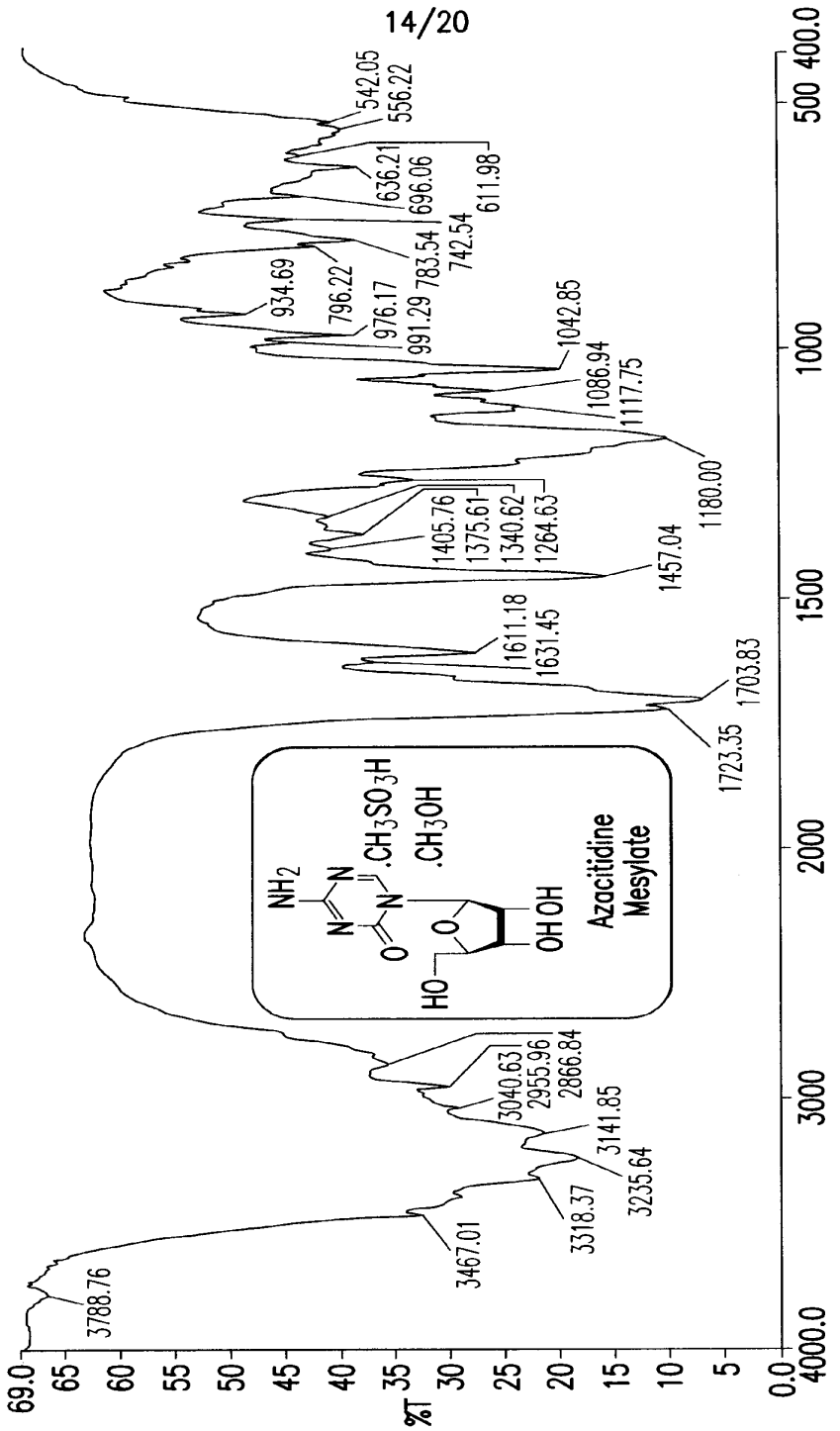

%T
cm-1
Azacitidine Mesylate
NH2
.CH3SO3H
.CH3OH
OH OH
HO
3788.76
3467.01
3318.37
3235.64
3141.85
3040.63
2955.96
2866.84
1723.35
1703.83
1631.45
1611.18
1457.04
1405.76
1375.61
1340.62
1264.63
1180.00
1117.75
1086.94
1042.85
991.29
976.17
934.69
796.22
783.54
742.54
696.06
636.21
611.98
556.22
542.05

FIG. 14

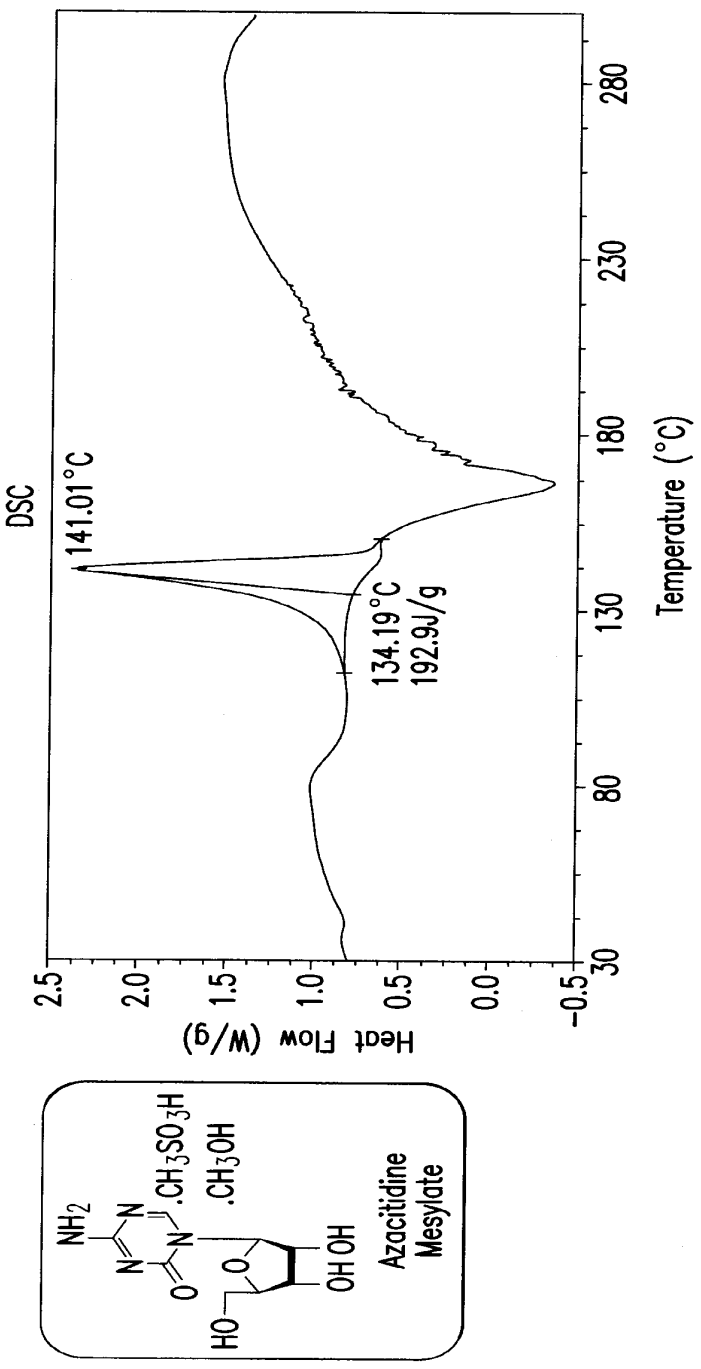

DSC
141.01°C
134.19°C
192.9J/g
Heat Flow (W/g)
Temperature (°C)
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
30
80
130
180
230
280
NH2
.CH3SO3H
.CH3OH
HO
OH OH
Azacitidine
Mesylate

FIG. 15

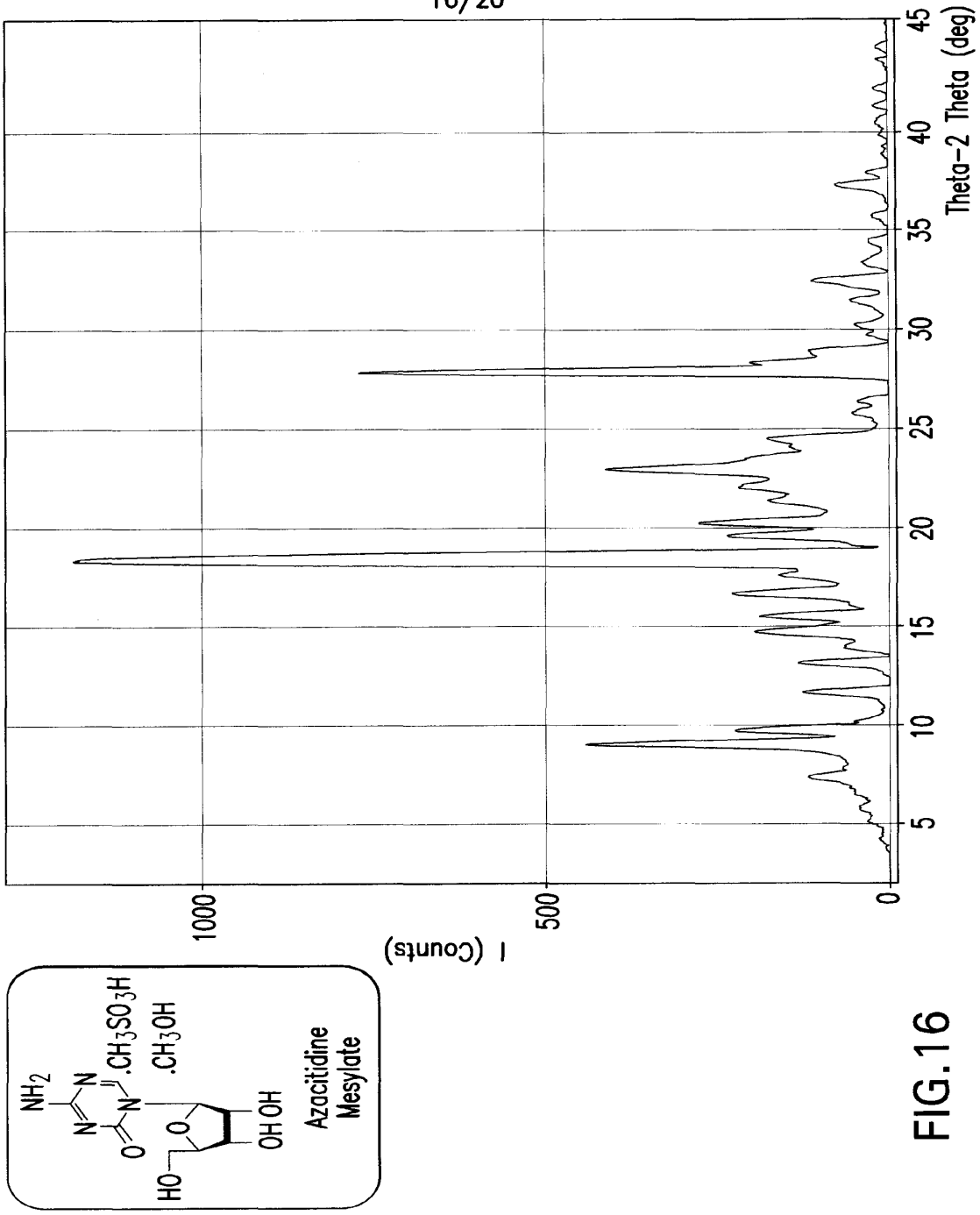


FIG.16

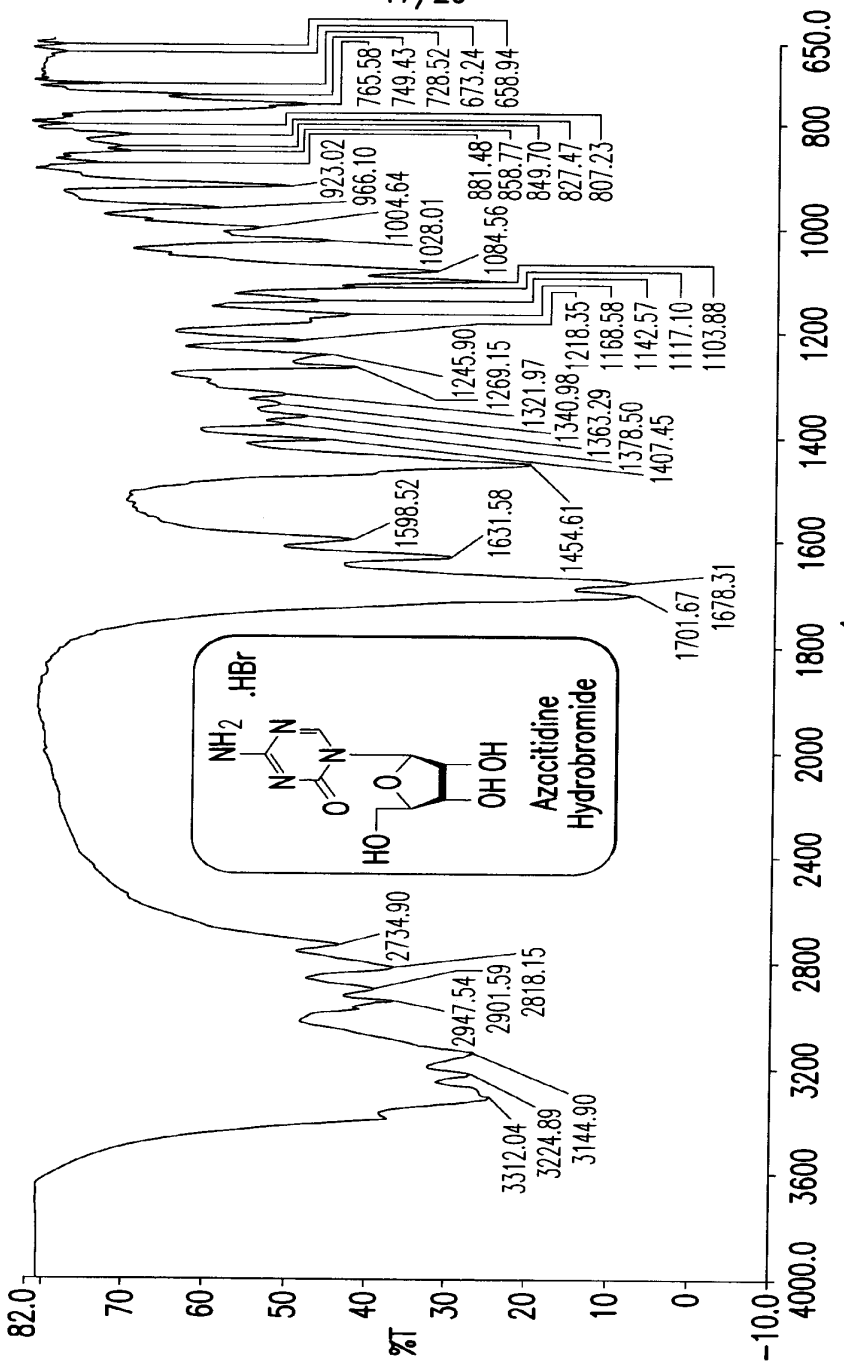


FIG.17

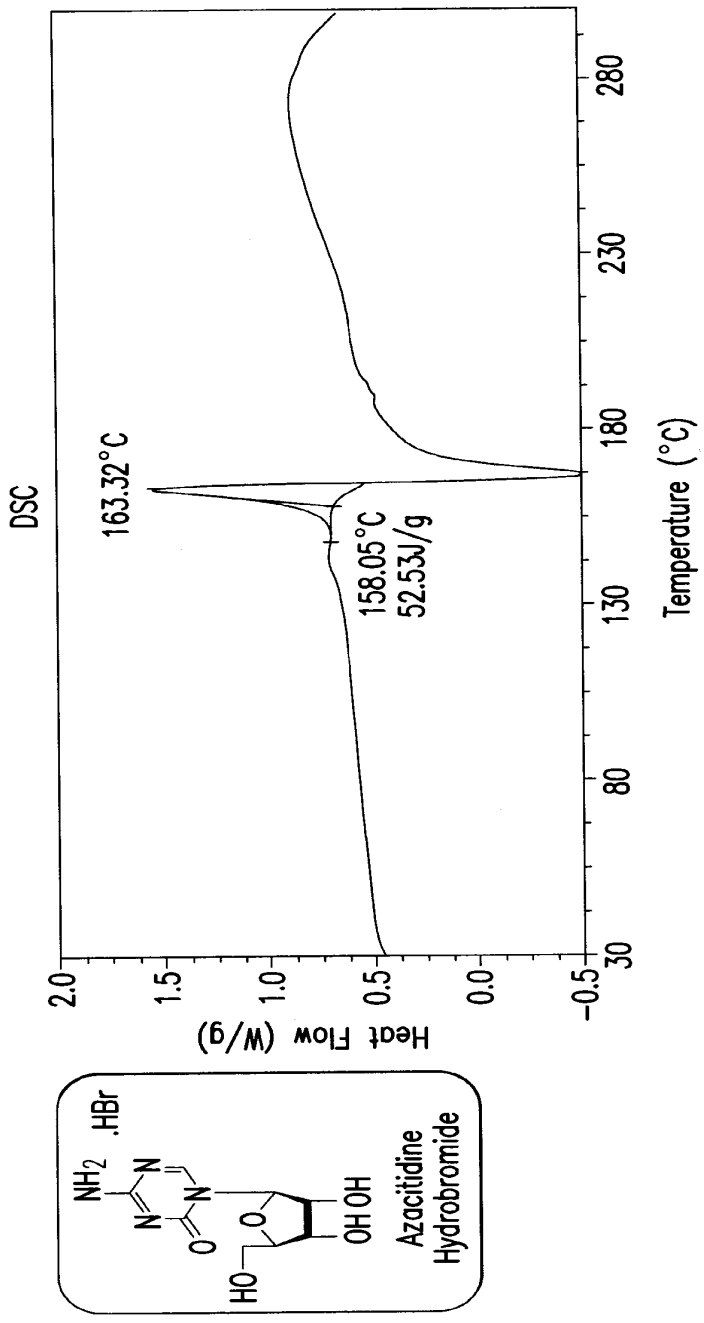


FIG.18

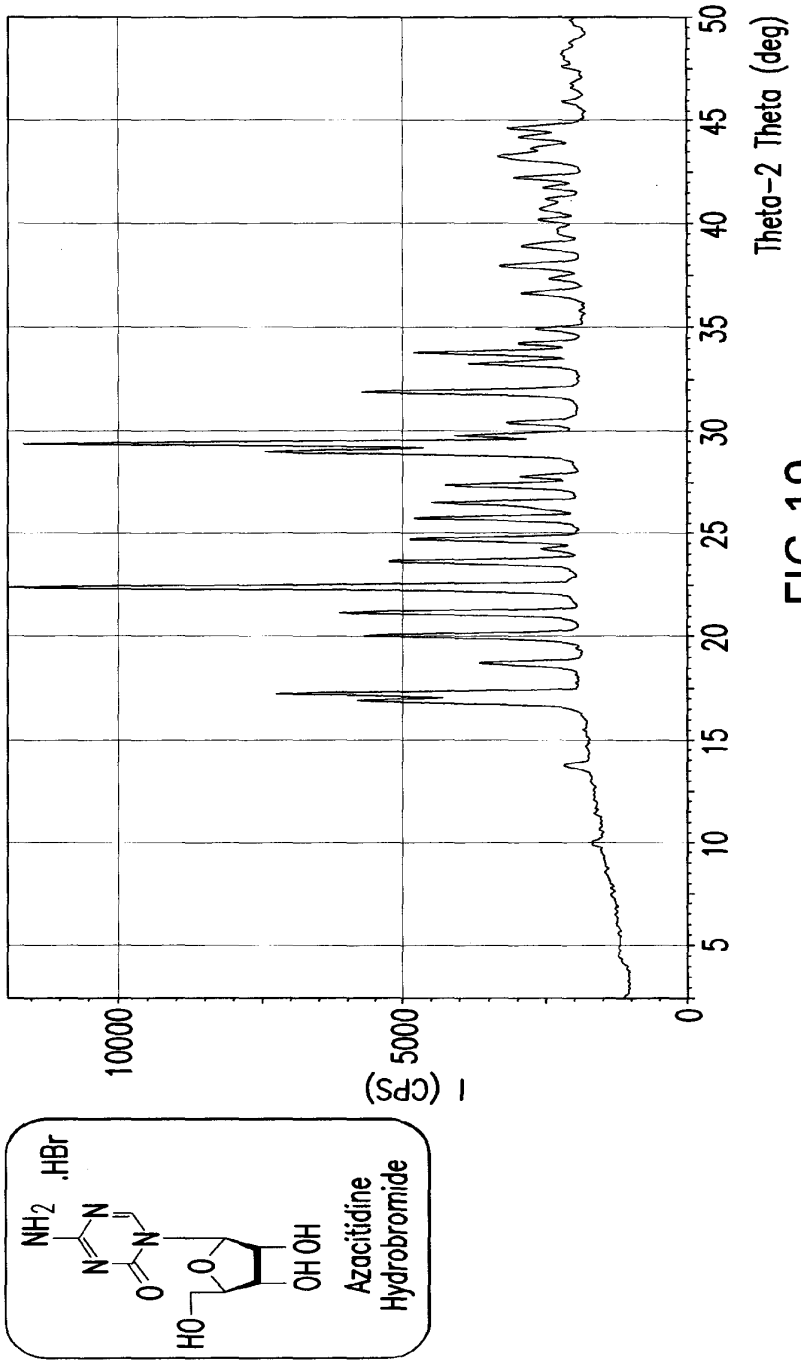

I (CPS)
10000
5000
0
5
10
15
20
25
30
35
40
45
50
Theta-2 Theta (deg)
NH2 .HBr
N
N
N
O
O
HO
OH OH
Azacitidine
Hydrobromide

FIG.19

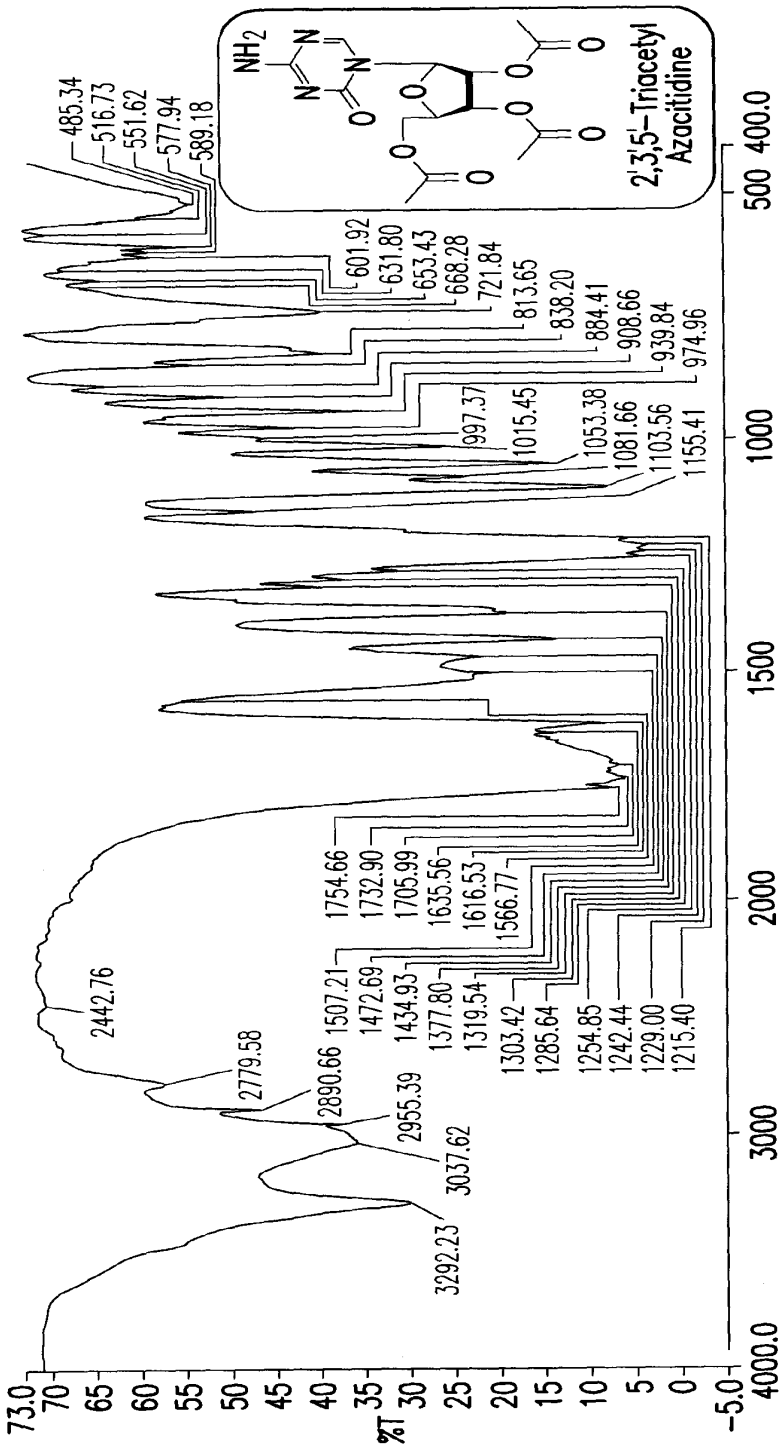


FIG.20

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2012/031059

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07H19/12
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, CHEM ABS Data, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	U. NIEDBALLA ET AL: "Synthesis of nucleosides. 13. General synthesis of N-glycosides. V. Synthesis of 5-azacytidines", THE JOURNAL OF ORGANIC CHEMISTRY, vol. 39, no. 25, 1 December 1974 (1974-12-01), pages 3672-3674, XP55011604, ISSN: 0022-3263, DOI: 10.1021/jo00939a012	1,2,47, 48
Y	the whole document	3-39
X	WO 2006/034154 A2 (SUPERGEN INC [US]; REDKAR SANJEEV [US]; PHIASIVONGSA PASIT [US]) 30 March 2006 (2006-03-30)	40,44-49
Y	page 12; compound 19 page 22 - page 25	3-39

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 July 2012	27/07/2012

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Nikolai, Joachim

7

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2012/031059

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2008/088779 A2 (IVAX PHARMACEUTICALS SRO [CZ]; TEVA PHARMA [US]; JEGOROV ALEXANDR [CZ]) 24 July 2008 (2008-07-24) Forms XI, XII; page 18 - page 21 example 18 -----	49
X	WO 2004/082619 A2 (PHARMION CORP [US]; ASH STEVENS INC [US]; IONESCU DUMITRU [US]; BLUMBE) 30 September 2004 (2004-09-30) example 1 page 6 - page 7; examples 6-8 -----	1,2,49

Form PCT/ISA/210 (continuation of second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2012/031059

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2006034154 A2	30-03-2006	AU 2005286910 A1	30-03-2006
		CA 2579687 A1	30-03-2006
		EP 1788874 A2	30-05-2007
		JP 2008513489 A	01-05-2008
		US 2006063735 A1	23-03-2006
		US 2010062992 A1	11-03-2010
		WO 2006034154 A2	30-03-2006
WO 2008088779 A2	24-07-2008	EP 2118117 A2	18-11-2009
		US 2008287378 A1	20-11-2008
		WO 2008088779 A2	24-07-2008
WO 2004082619 A2	30-09-2004	EP 1610784 A2	04-01-2006
		EP 2258710 A1	08-12-2010
		EP 2258711 A2	08-12-2010
		EP 2270022 A1	05-01-2011
		EP 2270023 A1	05-01-2011
		US 2004186065 A1	23-09-2004
		US 2005137150 A1	23-06-2005
		US 2006247189 A1	02-11-2006
		US 2010298253 A1	25-11-2010
		WO 2004082619 A2	30-09-2004

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-39, 47, 48

   A process for preparing 5-azacytidine or a salt, solvate, hydrate or polymorph thereof. 5-azacytidine or a salt, solvate, hydrate or polymorph thereof prepared by a process of claim 1.

   ---

2. claims: 40-46

   A salt of 5-azacytidine selected from the group consisting of hydrochloride salt, hydrobromide salt, sulfate salt.

   ---

3. claim: 49

   5-azacytidine or a salt, solvate of hydrate thereof which is substantially crystalline.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2012/031059

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☒ No protest accompanied the payment of additional search fees.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
14 February 2013 (14.02.2013)

WIPO | PCT

(10) International Publication Number
WO 2013/022872 A1

(51) **International Patent Classification**:
C12Q 1/68 (2006.01)

(21) **International Application Number**:
PCT/US2012/049826

(22) **International Filing Date**:
7 August 2012 (07.08.2012)

(25) **Filing Language**: English

(26) **Publication Language**: English

(30) **Priority Data**:
61/522,167 10 August 2011 (10.08.2011) US

(71) **Applicant** *(for all designated States except US)*: **CELGENE CORPORATION** [US/US]; 86 Morris Avenue, Summit, NJ 07901 (US).

(72) **Inventors; and**
(75) **Inventors/Applicants** *(for US only)*: **BEACH, C.L.** [US/US]; 520 NW Edgewood Trail, Lee's Summit, MO 64081 (US). **MACBETH, Kyle** [US/US]; 629 Broderick Street, San Francisco, CA 94117 (US). **SHI, Tao** [CN/US]; 13945 Gunnison Ct., San Diego, CA 92129 (US).

(74) **Agents**: **INSOGNA, Anthony, M.** et al.; Jones Day, 222 East 41st Street, New York, NY 10017-6702 (US).

(81) **Designated States** *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

WO 2013/022872 A1

(54) **Title**: GENE METHYLATION BIOMARKERS AND METHODS OF USE THEREOF

(57) **Abstract**: Provided herein are biomarkers, compositions, kits, and methods for diagnosis, prognosis, or monitoring of cancers and/or myelodysplasia syndromes (MDS), and for predicting or monitoring the efficacy of certain therapeutic treatment in cancer patients or in MDS patients, treated with a therapeutic agent, such as, a cytidine analog, *e.g.*, 5-azacytidine. Also provided are methods for predicting the overall survival of certain classes of patients having MDS or cancer, and methods for selecting patients having MDS or cancer for a particular therapeutic treatment. Also provided are gene methylation biomarkers and methods of use thereof.

GENE METHYLATION BIOMARKERS AND METHODS OF USE THEREOF

[0001] This application claims priority to U.S. Provisional Patent Application No. 61/522,167, filed August 10, 2011, which is hereby incorporated by reference in its entirety.

1. FIELD

[0002] Provided herein are biomarkers, compositions, kits, and methods, for diagnosis, prognosis, or monitoring of cancers and/or myelodysplastic syndromes (MDS), and for predicting or monitoring the efficacy or clinical benefit of certain therapeutic treatment in patients in need thereof, such as, in MDS patients treated with an agent, such as, a cytidine analog, *e.g.*, 5-azacytidine. Also provided are methods for selecting patients for a particular therapeutic treatment. Also provided are gene methylation biomarkers and methods of use thereof.

2. BACKGROUND

[0003] Cancer is a major worldwide public health problem; in the United States alone, approximately 560,000 people died of cancer in 2006. *See, e.g.*, U.S. Mortality Data 2006, National Center for Health Statistics, Centers for Disease Control and Prevention (2009). Many types of cancer have been described in the medical literature. Examples include cancer of blood, bone, skin, lung, colon, breast, prostate, ovary, brain, kidney, bladder, pancreas, and liver, among others. The incidence of cancer continues to climb as the general population ages and as new forms of cancer develop. A continuing need exists for effective therapies to treat subjects with cancer.

[0004] Myelodysplastic syndromes ("MDS") refers to a diverse group of hematopoietic stem cell disorders. MDS is characterized by a cellular marrow with impaired morphology and maturation (dysmyelopoiesis), peripheral blood cytopenias, and a variable risk of progression to acute leukemia, resulting from ineffective blood cell production. *See, e.g.*, *The Merck Manual* 953 (17th ed. 1999); List *et al.*, 1990, *J. Clin. Oncol.* 8:1424.

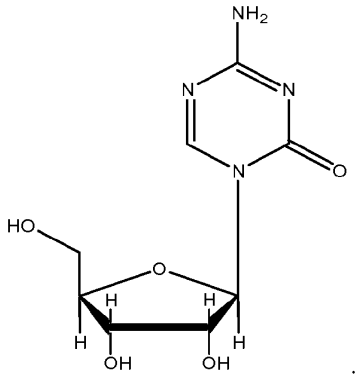
[0005] The initial hematopoietic stem cell injury can be from causes such as, but not limited to, cytotoxic chemotherapy, radiation, virus, chemical exposure, and genetic predisposition. A clonal mutation predominates over bone marrow, suppressing healthy stem cells. In the early stages of MDS, the main cause of cytopenias is increased programmed cell death (apoptosis). As the disease progresses and converts into leukemia, gene mutation

rarely occurs and a proliferation of leukemic cells overwhelms the healthy marrow. The disease course differs, with some cases behaving as an indolent disease and others behaving aggressively with a very short clinical course that converts into an acute form of leukemia.

[0006] An international group of hematologists, the French-American-British (FAB) Cooperative Group, classified MDS into five subgroups, differentiating them from acute myeloid leukemia. *See, e.g.*, *The Merck Manual* 954 (17th ed. 1999); Bennett J. M., *et al.*, *Ann. Intern. Med.* 1985 October, 103(4): 620-5; and Besa E. C., *Med. Clin. North Am.* 1992 May, 76(3): 599 617. An underlying trilineage dysplastic change in the bone marrow cells of the patients is found in all subtypes. Information is available regarding the pathobiology of MDS, certain MDS classification systems, and particular methods of treating and managing MDS. *See, e.g.*, U.S. Patent No. 7,189,740 (issued March 13, 2007), which is incorporated by reference herein in its entirety.

[0007] Nucleoside analogs have been used clinically for the treatment of viral infections and proliferative disorders for decades. Most of the nucleoside analog drugs are classified as antimetabolites. After they enter cells, nucleoside analogs are successively phosphorylated to nucleoside 5'-monophosphates, 5'-diphosphates, and 5'-triphosphates. In most cases, nucleoside triphosphates are the chemical entities that inhibit DNA or RNA synthesis, either through a competitive inhibition of polymerases or through incorporation of modified nucleotides into DNA or RNA sequences. Nucleosides may act also as their diphosphates.

[0008] 5-Azacytidine (also known as azacitidine and 4-amino-1-β-D-ribofuranosyl-1,3,5-triazin-2(1*H*)-one; Nation Service Center designation NSC-102816; CAS Registry Number 320-67-2) has undergone NCI-sponsored trials for the treatment of MDS. *See, e.g.*, Kornblith *et al.*, *J. Clin. Oncol.* 20(10): 2441-2452 (2002); Silverman *et al.*, *J. Clin. Oncol.* 20(10): 2429-2440 (2002). 5-Azacytidine may be defined as having a molecular formula of $C_8H_{12}N_4O_5$, a relative molecular weight of 244.21 and a structure of:



[0009] 5-Azacytidine (also referred to as azacitidine herein) is a nucleoside analog, more specifically a cytidine analog. 5-Azacytidine is an antagonist of its related natural nucleoside, cytidine. 5-Azacytidine, as well as decitabine, *i.e.*, 5-aza-2'-deoxycytidine, are antagonists of decitabine's related natural nucleoside, deoxycytidine. The only structural difference between the analogs and their related natural nucleosides is the presence of nitrogen at position 5 of the cytosine ring in place of carbon.

[0010] Other members of the class of cytidine analogs include, but are not limited to, arabinosylcytosine (Cytarabine), 2'-deoxy-2',2'-difluorocytidine (Gemcitabine), 5-aza-2'-deoxycytidine (Decitabine), 2(1*H*)-pyrimidine-riboside (Zebularine), 2',3'-dideoxy-5-fluoro-3'-thiacytidine (Emtriva), N^4-pentyloxycarbonyl-5'-deoxy-5-fluorocytidine (Capecitabine), 2'-cyclocytidine, arabinofuanosyl-5-azacytidine, dihydro-5-azacytidine, N^4-octadecyl-cytarabine, elaidic acid cytarabine, and cytosine 1-β-D-arabinofuranoside (ara-C).

[0011] A need remains for more effective methods, biomarkers, compositions, and kits, which provide, *e.g.*, increased survival to higher risk MDS patients.

[0012] Citation of any references in this Section of the application is not to be construed as an admission that such reference is prior art to the present application.

3. SUMMARY

[0013] In one embodiment, provided herein are biomarkers for diagnosis, prognosis, or monitoring of cancers and/or myelodysplastic syndromes (MDS). In one embodiment, the MDS is a higher risk MDS. In one embodiment, provided herein are biomarkers for predicting or monitoring the efficacy or clinical benefit of a therapeutic treatment in patients in need thereof, such as, in MDS patients treated with an agent, such as, a cytidine analog, *e.g.*, 5-azacytidine.

[0014] In one embodiment, provided herein are methods for diagnosis, prognosis, or monitoring of cancers and/or myelodysplastic syndromes (MDS). In one embodiment, the MDS is a higher risk MDS. In one embodiment, provided herein are methods for predicting or monitoring the efficacy or clinical benefit of a therapeutic treatment in patients in need thereof, such as, in MDS patients treated with an agent, such as, a cytidine analog, *e.g.*, 5-azacytidine.

[0015] In one embodiment, provided herein is a method of predicting or monitoring the efficacy or clinical benefit of a therapeutic treatment, comprising measuring the level of one or more specific biomarker(s) in cells obtained from patients having a certain disease before or during the treatment. In one embodiment, the disease is cancer. In one embodiment, the

cancer is a blood-borne tumor. In one embodiment, the cancer is a solid tumor. In one embodiment, the disease is MDS, *e.g.*, higher-risk MDS. In one embodiment, the treatment is administration of a cytidine analog provided herein. In one embodiment, the treatment is administration of 5-azacytidine. In one embodiment, provided herein is a method of predicting or monitoring the efficacy of 5-azacytidine in MDS patients (*e.g.*, in higher-risk MDS patients), comprising measuring the level of one or more specific biomarker(s) in cells obtained from patients before or during 5-azacytidine treatment. In one embodiment, the cells are obtained from the bone marrow of patient(s). In one embodiment, the biomarker provided herein is methylation of one or more gene(s). In one embodiment, the biomarker provided herein is methylation of one or more gene(s) at one or more locus/loci (*e.g.*, at particular CpG site(s)). In one embodiment, the biomarker provided herein is a methylation pattern or a methylation signature of a particular group of genes. In one embodiment, the biomarker provided herein is a methylation pattern or a methylation signature of a particular group of genes at particular loci. In one embodiment, the gene methylation biomarkers provided herein include, but are not limited to, methylation of one or more of the following genes: *CDKN2B (p15)*, *SOCS1*, *CDH1 (E-cadherin)*, *TP73*, and/or *CTNNA1 (α-catenin)*. In one embodiment, the gene methylation biomarkers provided herein include, but are not limited to, methylation of one or more of the following genes: *ABHD14A*, *ABO*, *ADAMTS18*, *ADRA2B*, *ADRB3*, *AIRE*, *AKAP12*, *ALOX15B*, *ALS2CR11*, *AMT*, *ANKRD33*, *APC2*, *AVP*, *BHMT*, *C18orf22*, *C19orf30*, *C1orf172*, *C1orf87*, *C3orf15*, *C1QTNF6*, *C22orf27*, *C7orf16*, *C7orf41*, *CBX7*, *CCDC19*, *CCDC81*, *CD164L2*, *CDH1*, *CDKN2B*, *CHAD*, *CHRNG*, *CIDEB*, *CKMT1B*, *CKMT2*, *CLCN6*, *CLDN6*, *CLDN9*, *CNTN4*, *CPT1B*, *CRHBP*, *CXCL5*, *CYP2E1*, *CYP26C1*, *DES*, *DPYS*, *DYDC1*, *EGFL7*, *ELMO3*, *ENTPD2*, *ENTPD3*, *ESR1*, *EYA4*, *F2RL2*, *FAM57B*, *FBLN1*, *FBXO2*, *FKBP1B*, *FLJ44881*, *FLVCR2*, *FREQ*, *FZD9*, *GAB1*, *GAS2L2*, *GATA4*, *GBGT1*, *GDF5*, *GHSR*, *GNAS*, *GNMT*, *GNPNAT1*, *GP1BA*, *GPR25*, *GRM6*, *GSTM5*, *HCN4*, *HIST1H1A*, *HOXD4*, *HSPA2*, *HTATIP2*, *HTR7*, *HYDIN*, *IGDCC3*, *ILDR1*, *IRF6*, *KAZALD1*, *KCNA6*, *KCNK3*, *KCNQ1*, *KIAA0427*, *KIR3DX1*, *KRT25*, *KRT7*, *KRT72*, *LAD1*, *LAMA4*, *LAMC2*, *LGTN*, *LRRC17*, *LTF*, *MBD3L1*, *MEGF10*, *MICAL1*, *MRPL28*, *MTMR9*, *MTNR1B*, *NALCN*, *NCAN*, *NCOR2*, *NDRG2*, *NDUFAF3*, *NEUROG1*, *NGB*, *NPFFR2*, *NPM2*, *NPPB*, *NPR2*, *NXN*, *OBFC2B*, *OGFR*, *ONECUT2*, *OTOP1*, *OXT*, *PACSIN1*, *PAOX*, *PARP3*, *PAX1*, *PCDH8*, *PCDHAC2*, *PDE4C*, *PF4V1*, *PKDREJ*, *PM20D1*, *POMC*, *POU3F1*, *PPAPDC3*, *PRIC285*, *PRLH*, *PSMD11*, *PTGIS*, *RAB36*, *RAP1GAP*, *RASGRF1*, *RASIP1*, *RBPJL*, *RLN1*, *RPL36*, *RPL36AL*, *RPUSD3*, *SCG5*, *SCMH1*, *SCUBE3*, *SEMA3B*, *SGPP2*, *SHROOM1*, *SKAP1*, *SLC12A8*, *SLC5A8*, *SNN*, *SORBS3*, *SPG7*, *SPINT1*,

SRD5A2, *SRRT*, *SSTR4*, *STMN1*, *TBC1D1*, *TCEA2*, *TCF15*, *TFAP2E*, *TGFBI*, *TIAM1*, *TMEM125*, *TMEM151A*, *TMEM184A*, *TMEM189*, *TMOD3*, *TNNT1*, *TP53INP1*, *TRPC4*, *TRPM3*, *UNC80*, *VAMP5*, *VHL*, *VSTM1*, *WBSCR27*, *WDR52*, *WT1*, *ZFP41*, *ZNF205*, and/or *ZNF710*. In one embodiment, the gene methylation biomarkers provided herein include, but are not limited to, methylation of one or more of the following genes: *WT1*, *CDKN2B*, and *CDH1*. In one embodiment, the gene methylation biomarkers provided herein include, but are not limited to, methylation of one or more of the following gene: *WT1*. In one embodiment, the gene methylation biomarker provided herein include the methylation of one or more gene(s) provided herein at particular locus/loci. In one embodiment, the biomarkers provided herein can be used to predict whether a particular therapeutic intervention is likely to be successful in treating a cancer. In one embodiment, the biomarkers provided herein can be used to predict whether a particular therapeutic intervention is likely to be successful in treating MDS, *e.g.*, higher-risk MDS. Further, the biomarkers provided herein can be used to monitor efficacy or progress of a drug treatment once the treatment begins.

[0016] In one embodiment, provided herein are methods for predicting the overall survival of certain classes of patients having MDS, *e.g.*, after a therapeutic treatment. In one embodiment, the MDS is higher-risk MDS. In one embodiment, the method comprises the step of measuring the methylation level(s) of one or more gene(s) in cells obtained from patients. In one embodiment, the method comprises the step of measuring the methylation level(s) of one or more gene(s) at particular locus/loci in cells obtained from patients. In one embodiment, the method further comprises the step of grouping patients based on methylation level(s) of one or more gene(s). In one embodiment, the method further comprises the step of grouping patients based on methylation level(s) of one or more gene(s) at particular locus/loci. In one embodiment, the method further comprises the step of selecting patients for a particular therapeutic treatment based on methylation level(s) of one or more gene(s). In one embodiment, the method further comprises the step of selecting patients for a particular therapeutic treatment based on methylation level(s) of one or more gene(s) at particular locus/loci. In one embodiment, the method further comprises the step of treating certain groups or classes of patients with a therapeutic agent. In one embodiment, the treatment is administration of a cytidine analog provided herein. In one embodiment, the treatment is administration of 5-azacytidine. In one embodiment, the patients selected based on methylation level(s) of one or more gene(s) (*e.g.*, at particular locus/loci) exhibit better or prolonged overall survival after the therapeutic treatment. In one embodiment, the patients

selected based on methylation level(s) of one or more gene(s) (*e.g.*, at particular locus/loci) exhibit better or prolonged time to AML transformation after the therapeutic treatment.

[0017] Also provided are gene methylation biomarkers and methods of use thereof. In one embodiment, provided herein is a methylation pattern or methylation signature of a group of genes that is predictive of therapeutic efficacy or clinical benefit of a particular therapeutic agent in treating cancer. In one embodiment, provided herein is a methylation pattern or methylation signature of a group of genes that is predictive of therapeutic efficacy or clinical benefit of a particular therapeutic agent in treating MDS. In specific embodiments, provided herein is a methylation pattern or methylation signature of a group of genes that is predictive of overall survival of patients having MDS after receiving 5-azacytidine treatment. In specific embodiments, provided herein is a methylation pattern or methylation signature of a group of genes that is predictive of time to AML transformation in patients having MDS after receiving 5-azacytidine treatment. In one embodiment, the gene methylation biomarker provided herein involves the methylation of at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, at least 25, at least 30, at least 35, at least 40, at least 45, at least 50, at least 55, at least 60, at least 65, at least 70, at least 75, at least 80, at least 85, at least 90, at least 95, at least 100, at least 110, at least 120, at least 130, at least 140, at least 150, at least 160, at least 170, at least 180, or at least 190 genes provided herein elsewhere. In one embodiment, the gene methylation biomarker provided herein involves the methylation level(s) of about 1, about 2, about 3, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, about 18, about 19, about 20, about 25, about 30, about 35, about 40, about 45, about 50, about 55, about 60, about 65, about 70, about 75, about 80, about 85, about 90, about 95, about 100, about 110, about 120, about 130, about 140, about 150, about 160, about 170, about 180, or about 190 genes provided herein elsewhere. In one embodiment, the gene methylation biomarker provided herein involves the methylation level(s) of up to 1, up to 2, up to 3, up to 4, up to 5, up to 6, up to 7, up to 8, up to 9, up to 10, up to 11, up to 12, up to 13, up to 14, up to 15, up to 16, up to 17, up to 18, up to 19, up to 20, up to 25, up to 30, up to 35, up to 40, up to 45, up to 50, up to 55, up to 60, up to 65, up to 70, up to 75, up to 80, up to 85, up to 90, up to 95, up to 100, up to 110, up to 120, up to 130, up to 140, up to 150, up to 160, up to 170, up to 180, or up to 190 genes provided herein elsewhere. In one embodiment, the gene methylation biomarker provided herein involves the methylation level(s) of one or more gene cluster(s) provided herein. In one

embodiment, the gene methylation biomarker provided herein involves the methylation level(s) of one or more genes (*e.g.*, at particular locus/loci) selected from the group consisting of: *ABHD14A*, *ABO*, *ADAMTS18*, *ADRA2B*, *ADRB3*, *AIRE*, *AKAP12*, *ALOX15B*, *ALS2CR11*, *AMT*, *ANKRD33*, *APC2*, *AVP*, *BHMT*, *C18orf22*, *C19orf30*, *C1orf172*, *C1orf87*, *C3orf15*, *C1QTNF6*, *C22orf27*, *C7orf16*, *C7orf41*, *CBX7*, *CCDC19*, *CCDC81*, *CD164L2*, *CDH1*, *CDKN2B*, *CHAD*, *CHRNG*, *CIDEB*, *CKMT1B*, *CKMT2*, *CLCN6*, *CLDN6*, *CLDN9*, *CNTN4*, *CPT1B*, *CRHBP*, *CXCL5*, *CYP2E1*, *CYP26C1*, *DES*, *DPYS*, *DYDC1*, *EGFL7*, *ELMO3*, *ENTPD2*, *ENTPD3*, *ESR1*, *EYA4*, *F2RL2*, *FAM57B*, *FBLN1*, *FBXO2*, *FKBP1B*, *FLJ44881*, *FLVCR2*, *FREQ*, *FZD9*, *GAB1*, *GAS2L2*, *GATA4*, *GBGT1*, *GDF5*, *GHSR*, *GNAS*, *GNMT*, *GNPNAT1*, *GP1BA*, *GPR25*, *GRM6*, *GSTM5*, *HCN4*, *HIST1H1A*, *HOXD4*, *HSPA2*, *HTATIP2*, *HTR7*, *HYDIN*, *IGDCC3*, *ILDR1*, *IRF6*, *KAZALD1*, *KCNA6*, *KCNK3*, *KCNQ1*, *KIAA0427*, *KIR3DX1*, *KRT25*, *KRT7*, *KRT72*, *LAD1*, *LAMA4*, *LAMC2*, *LGTN*, *LRRC17*, *LTF*, *MBD3L1*, *MEGF10*, *MICAL1*, *MRPL28*, *MTMR9*, *MTNR1B*, *NALCN*, *NCAN*, *NCOR2*, *NDRG2*, *NDUFAF3*, *NEUROG1*, *NGB*, *NPFFR2*, *NPM2*, *NPPB*, *NPR2*, *NXN*, *OBFC2B*, *OGFR*, *ONECUT2*, *OTOP1*, *OXT*, *PACSIN1*, *PAOX*, *PARP3*, *PAX1*, *PCDH8*, *PCDHAC2*, *PDE4C*, *PF4V1*, *PKDREJ*, *PM20D1*, *POMC*, *POU3F1*, *PPAPDC3*, *PRIC285*, *PRLH*, *PSMD11*, *PTGIS*, *RAB36*, *RAP1GAP*, *RASGRF1*, *RASIP1*, *RBPJL*, *RLN1*, *RPL36*, *RPL36AL*, *RPUSD3*, *SCG5*, *SCMH1*, *SCUBE3*, *SEMA3B*, *SGPP2*, *SHROOM1*, *SKAP1*, *SLC12A8*, *SLC5A8*, *SNN*, *SORBS3*, *SPG7*, *SPINT1*, *SRD5A2*, *SRRT*, *SSTR4*, *STMN1*, *TBC1D1*, *TCEA2*, *TCF15*, *TFAP2E*, *TGFBI*, *TIAM1*, *TMEM125*, *TMEM151A*, *TMEM184A*, *TMEM189*, *TMOD3*, *TNNT1*, *TP53INP1*, *TRPC4*, *TRPM3*, *UNC80*, *VAMP5*, *VHL*, *VSTM1*, *WBSCR27*, *WDR52*, *WT1*, *ZFP41*, *ZNF205*, and *ZNF710*.

[0018] In particular embodiments, provide herein are methods for the treatment, prevention, and/or management of cancer using compositions comprising an effective amount of a cytidine analog, including, but not limited to, 5-azacytidine. In certain embodiments, the methods comprise treating, preventing, and/or managing certain types of cancer, including, but not limited to, blood-borne tumor or solid tumor. In certain embodiments, the methods comprise co-administering two or more active agents. In certain embodiments, the methods comprise treating, preventing, and/or managing cancer using one or more of the methods provided herein, together with one or more of the treatments including chemotherapy, immunotherapy, targeted therapy, and/or radiation therapy.

[0019] In particular embodiments, provide herein are methods for the treatment, prevention, and/or management of MDS using compositions comprising an effective amount of a cytidine analog, including, but not limited to, 5-azacytidine. In certain embodiments, the

methods comprise treating, preventing, and/or managing certain types of MDS, including, but not limited to, higher-risk MDS. In certain embodiments, the methods comprise co-administering two or more active agents. In certain embodiments, the methods comprise treating, preventing, and/or managing MDS using one or more of the methods provided herein, together with one or more of the treatments including chemotherapy, immunotherapy, targeted therapy, and/or radiation therapy.

[0020] Particular embodiments provide methods for treating patients with higher risk MDS using 5-azacytidine. Particular embodiments provide methods for improving the overall survival of patients having MDS, *e.g.*, higher risk MDS. Particular embodiments provide methods for selecting patients having better expected response to a treatment. Particular embodiments provide alternative dosing regimens for treating MDS. Particular embodiments provide methods for treating certain subgroups of patients with higher risk MDS, *e.g.*, patients with -7/del(7q) and/or patients with a particular gene methylation profile (or gene methylation pattern or gene methylation signature) prior to a therapeutic treatment or after initiation of a therapeutic treatment. Particular embodiments provide methods for treating elderly patients with acute myelogenous leukemia ("AML"). Particular embodiments provide methods for ameliorating certain adverse events ("AEs") in patients with MDS, *e.g.*, higher risk MDS. Particular embodiments provide methods for treating patients having MDS, *e.g.*, higher risk MDS, using specific numbers of 5-azacytidine treatment cycles. Particular embodiments provide methods of treating patients who meet the WHO criteria for AML using 5-azacytidine. Particular embodiments provide methods of using IWG responses of complete remission, partial remission, hematologic improvement, and/or stable disease as predictors of overall response in patients with MDS, *e.g.*, higher risk MDS. Particular embodiments provide using 5-azacytidine as maintenance therapy. Particular embodiments provide using DNA and/or RNA methylation as biomarkers for overall survival in patients with MDS, *e.g.*, higher risk MDS.

[0021] In one embodiment, the cytidine analog described herein includes, but is not limited to, 5-aza-2'-deoxycytidine, 5-azacytidine, 5-aza-2'-deoxy-2',2'-difluorocytidine, 5-aza-2'-deoxy-2'-fluorocytidine, 2'-deoxy-2',2'-difluorocytidine, cytosine 1-β-D-arabinofuranoside, 2(1H) pyrimidine riboside, 2'-cyclocytidine, arabinofuanosyl-5-azacytidine, dihydro-5-azacytidine, N^4-octadecyl-cytarabine, and elaidic acid cytarabine.

[0022] In one embodiment, the cytidine analog is administered parenterally (*e.g.*, intravenously or subcutaneously). In one embodiment, the cytidine analog is administered

orally. In specific embodiments, 5-azacytidine is administered parenterally (*e.g.*, intravenously or subcutaneously). In specific embodiments, 5-azacytidine is administered orally. In specific embodiments, 5-azacytidine is administered in an amount of between about 75 mg/m^2 to about 100 mg/m^2 per day, *e.g.*, for up to about 7 consecutive days followed by a resting period of about 21 days (*e.g.*, a 28-day treatment cycle). In specific embodiments, 5-azacytidine is administered for at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or greater than 20 cycles. In one embodiment, the starting dose of a 5-azacytidine treatment is about 75 mg/m^2 per day, *e.g.*, for about 7 days followed by a resting period of about 21 days (a 28-day treatment cycle), *e.g.*, for two 28-day treatment cycles. In one embodiment, after two 28-day treatment cycles, 5-azacytidine is administered in an amount of between about 75 mg/m^2 to about 100 mg/m^2 per day, *e.g.*, for up to about 7 consecutive days followed by a resting period of up to about 21 days (*e.g.*, a 28-day treatment cycle), *e.g.*, for at least two, at least three, at least four, at least five, or at least six additional 28-day treatment cycles. Certain embodiments herein provide co-administration of a cytidine analog (*e.g.*, 5-azacytidine) with one or more additional active agents to provide a synergistic therapeutic effect in subjects in need thereof. The co-administered agent(s) may be a cancer therapeutic agent, as described herein. In certain embodiments, the co-administered agent(s) may be dosed, *e.g.*, orally or by injection (*e.g.*, intravenous or subcutaneous injection).

[0023] In one embodiment, provided herein are compositions and kits for diagnosis, prognosis, or monitoring of cancers and/or myelodysplastic syndromes (MDS), *e.g.*, higher risk MDS. In one embodiment, provided herein are compositions and kits for predicting or monitoring the efficacy of a therapeutic treatment in patients having a certain disease (*e.g.*, a cancer or MDS), such as, in MDS patients treated with a cytidine analog, e.g., 5-azacytidine.

4. BRIEF DESCRIPTION OF THE DRAWINGS

[0024] Figure 1 represents a study design for the Phase III 5-azacytidine survival study.

[0025] Figure 2 represents a graph showing overall survival in the intent to treat population (higher risk MDS patients) of 5-azacytidine compared to conventional care regimens.

[0026] Figure 3 represents time to transform to AML – ITT Population comparing the 5-azacytidine group with the CCR group, showing difference of 13.7 months in time to transformation.

[0027] Figure 4 represents a correlation of baseline CDH1 methylation with overall survival or with time to AML transformation, in MDS patients treated with 5-azacytidine or with conventional care regimens.

[0028] Figure 5 represents a dosing and sample collection schedule for a clinical study of MDS patients treated with 5-azacytidine.

[0029] Figure 6 represents a baseline gene methylation pattern (*e.g.*, pre-treatment gene methylation pattern) in a training data set of 38 MDS patients, in relation to various clinical outcomes, including overall survival, after treatment of 5-azacytidine.

[0030] Figure 7 represents a baseline gene methylation pattern (*e.g.*, pre-treatment gene methylation pattern) in a data set of 59 MDS patients, in relation to various clinical outcomes, including overall survival, after treatment with 5-azacytidine.

5. DETAILED DESCRIPTION

[0031] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art. All publications and patents referred to herein are incorporated by reference herein in their entireties.

5.1 Definitions

[0032] As used in the specification and the accompanying claims, the indefinite articles "a" and "an" and the definite article "the" include plural as well as singular referents, unless the context clearly dictates otherwise.

[0033] As used herein, and unless otherwise specified, the term "about" or "approximately" means an acceptable error for a particular value as determined by one of ordinary skill in the art, which depends in part on how the value is measured or determined. In certain embodiments, the term "about" or "approximately" means within 1, 2, 3, or 4 standard deviations. In certain embodiments, the term "about" or "approximately" means within 30%, 25%, 20%, 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.1%, or 0.05% of a given value or range.

[0034] As used herein, and unless otherwise specified, the terms "treat," "treating" and "treatment" refer to the eradication or amelioration of a disease or disorder, or of one or more symptoms associated with the disease or disorder. In certain embodiments, the terms refer to minimizing the spread or worsening of the disease or disorder resulting from the administration of one or more prophylactic or therapeutic agents to a subject with such a disease or disorder. In some embodiments, the terms refer to the administration of a

compound or dosage form provided herein, with or without one or more additional active agent(s), after the diagnosis or the onset of symptoms of the particular disease.

[0035] As used herein, and unless otherwise specified, the terms "prevent," "preventing" and "prevention" refer to the prevention of the onset, recurrence or spread of a disease or disorder, or of one or more symptoms thereof. In certain embodiments, the terms refer to the treatment with or administration of a compound or dosage form provided herein, with or without one or more other additional active agent(s), prior to the onset of symptoms, particularly to subjects at risk of disease or disorders provided herein. The terms encompass the inhibition or reduction of a symptom of the particular disease. In certain embodiments, subjects with familial history of a disease are potential candidates for preventive regimens. In certain embodiments, subjects who have a history of recurring symptoms are also potential candidates for prevention. In this regard, the term "prevention" may be interchangeably used with the term "prophylactic treatment."

[0036] As used herein, and unless otherwise specified, the terms "manage," "managing" and "management" refer to preventing or slowing the progression, spread or worsening of a disease or disorder, or of one or more symptoms thereof. Often, the beneficial effects that a subject derives from a prophylactic and/or therapeutic agent do not result in a cure of the disease or disorder. In this regard, the term "managing" encompasses treating a subject who had suffered from the particular disease in an attempt to prevent or minimize the recurrence of the disease.

[0037] As used herein, and unless otherwise specified, "amelioration" of the symptoms of a particular disorder by administration of a particular pharmaceutical composition refers to any lessening, whether permanent or temporary, lasting or transient, that can be attributed to or associated with the administration of the composition.

[0038] As used herein, and unless otherwise specified, the term "therapeutically effective amount" or "effective amount" of a compound means an amount sufficient to provide a therapeutic benefit in the treatment or management of a disease or disorder, or to delay or minimize one or more symptoms associated with the disease or disorder. A "therapeutically effective amount" or "effective amount" of a compound means an amount of therapeutic agent, alone or in combination with one or more other agent(s), which provides a therapeutic benefit in the treatment or management of the disease or disorder. The terms "therapeutically effective amount" and "effective amount" can encompass an amount that improves overall therapy, reduces, delays, or avoids symptoms or causes of disease or disorder, or enhances the therapeutic efficacy of another therapeutic agent.

[0039] As used herein, and unless otherwise specified, a "prophylactically effective amount" of a compound is an amount sufficient to prevent a disease or disorder, or prevent its recurrence. A prophylactically effective amount of a compound means an amount of therapeutic agent, alone or in combination with one or more other agent(s), which provides a prophylactic benefit in the prevention of the disease. The term "prophylactically effective amount" can encompass an amount that improves overall prophylaxis or enhances the prophylactic efficacy of another prophylactic agent.

[0040] As used herein, and unless otherwise specified, the term "subject" is defined herein to include animals such as mammals, including, but not limited to, primates (*e.g.*, humans), cows, sheep, goats, horses, dogs, cats, rabbits, rats, mice, and the like. In specific embodiments, the subject is a human. The terms "subject" and "patient" are used interchangeably herein in reference, for example, to a mammalian subject, such as a human. In particular embodiments, a subject having MDS is a subject who has been previously diagnosed as having MDS.

[0041] As used herein, and unless otherwise specified, "tumor" refers to all neoplastic cell growth and proliferation, whether malignant or benign, and all pre-cancerous and cancerous cells and tissues. As used herein, and unless otherwise specified, "neoplastic" refers to any form of dysregulated or unregulated cell growth, whether malignant or benign, resulting in abnormal tissue growth. Thus, "neoplastic cells" include malignant and benign cells having dysregulated or unregulated cell growth.

[0042] As used herein, and unless otherwise specified, the terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include, but are not limited to, lymphoma, leukemia, and solid tumors, such as, for example, lung cancer. In one embodiment, the term "cancer" as used herein includes, but is not limited to, solid tumors and blood-borne tumors. The term "cancer" refers to disease of skin tissues, organs, blood, and vessels, including, but not limited to, cancers of the bladder, bone or blood, brain, breast, cervix, chest, colon, endometrium, esophagus, eye, head, kidney, liver, lymph nodes, lung, mouth, neck, ovaries, pancreas, prostate, rectum, stomach, testis, throat, and uterus. Specific cancers include, but are not limited to, advanced malignancy, amyloidosis, neuroblastoma, meningioma, atypical meningioma, hemangiopericytoma, multiple brain metastase, glioblastoma multiforms, glioblastoma, brain stem glioma, poor prognosis malignant brain tumor, malignant glioma, recurrent malignant glioma, anaplastic astrocytoma, anaplastic oligodendroglioma, neuroendocrine tumor, rectal adenocarcinoma, Dukes C & D colorectal

cancer, unresectable colorectal carcinoma, metastatic hepatocellular carcinoma, Kaposi's sarcoma, karyotype acute myeloblastic leukemia, Hodgkin's lymphoma, non-Hodgkin's lymphoma, cutaneous T-Cell lymphoma, cutaneous B-Cell lymphoma, diffuse large B-Cell lymphoma, low grade follicular lymphoma, metastatic melanoma (localized melanoma, including, but not limited to, ocular melanoma), malignant mesothelioma, malignant pleural effusion mesothelioma syndrome, peritoneal carcinoma, papillary serous carcinoma, gynecologic sarcoma, soft tissue sarcoma, scelroderma, cutaneous vasculitis, Langerhans cell histiocytosis, leiomyosarcoma, fibrodysplasia ossificans progressiva, hormone refractory prostate cancer, resected high-risk soft tissue sarcoma, unrescectable hepatocellular carcinoma, Waldenstrom's macroglobulinemia, smoldering myeloma, indolent myeloma, fallopian tube cancer, androgen independent prostate cancer, androgen dependent stage IV non-metastatic prostate cancer, hormone-insensitive prostate cancer, chemotherapy-insensitive prostate cancer, papillary thyroid carcinoma, follicular thyroid carcinoma, medullary thyroid carcinoma, and leiomyoma. In a specific embodiment, the cancer is metastatic. In another embodiment, the cancer is refractory or resistant to chemotherapy or radiation.

[0043] As used herein, and unless otherwise specified, the term "proliferative" disorder or disease refers to unwanted cell proliferation of one or more subset of cells in a multicellular organism resulting in harm (*i.e.,* discomfort or decreased life expectancy) to the multicellular organism. For example, as used herein, proliferative disorder or disease includes neoplastic disorders and other proliferative disorders.

[0044] As used herein, and unless otherwise specified, the term "relapsed" refers to a situation where a subject, that has had a remission of cancer after a therapy, has a return of cancer cells.

[0045] As used herein, and unless otherwise specified, the term "refractory" or "resistant" refers to a circumstance where a subject, even after intensive treatment, has residual cancer cells in the body.

[0046] As used herein, and unless otherwise specified, the term "drug resistance" refers to the condition when a disease does not respond to the treatment of a drug or drugs. Drug resistance can be either intrinsic, which means the disease has never been responsive to the drug or drugs, or it can be acquired, which means the disease ceases responding to a drug or drugs that the disease had previously responded to. In certain embodiments, drug resistance is intrinsic. In certain embodiments, the drug resistance is acquired.

[0047] As used herein, and unless otherwise specified, the term "anticancer agent" or "cancer therapeutic agent" is meant to include anti-proliferative agents and chemotherapeutic agents, including, but not limited to, antimetabolites (*e.g.*, 5-fluoro uracil, methotrexate, fludarabine, cytarabine (also known as cytosine arabinoside or Ara-C), and high dose cytarabine), antimicrotubule agents (*e.g.*, vinca alkaloids, such as vincristine and vinblastine; and taxanes, such as paclitaxel and docetaxel), alkylating agents (*e.g.*, mechlorethamine, chlorambucil, cyclophosphamide, melphalan, melphalan, ifosfamide, carmustine, azacitidine, decitabine, busulfan, cyclophosphamide, dacarbazine, ifosfamide, and nitrosoureas, such as carmustine, lomustine, bischloroethylnitrosurea, and hydroxyurea), platinum agents (*e.g.*, cisplatin, carboplatin, oxaliplatin, satraplatin (JM-216), and CI-973), anthracyclines (*e.g.*, doxorubicin and daunorubicin), antitumor antibiotics (*e.g.*, mitomycin, bleomycin, idarubicin, adriamycin, daunomycin (also known as daunorubicin, rubidomycin, or cerubidine), and mitoxantrone), topoisomerase inhibitors (*e.g.*, etoposide and camptothecins), purine antagonists or pyrimidine antagonists (*e.g.*, 6-mercaptopurine, 5-fluorouracil, cytarabine, clofarabine, and gemcitabine), cell maturing agents (*e.g.*, arsenic trioxide and tretinoin), DNA repair enzyme inhibitors (*e.g.*, podophyllotoxines, etoposide, irinotecan, topotecan, and teniposide), enzymes that prevent cell survival (*e.g.*, asparaginase and pegaspargase), histone deacetylase inhibitors (*e.g.*, vorinostat), any other cytotoxic agents (*e.g.*, estramustine phosphate, dexamethasone, prednimustine, and procarbazine), hormones (*e.g.*, dexamethasone, prednisone, methylprednisolone, tamoxifen, leuprolide, flutamide, and megestrol), monoclonal antibodies (*e.g.*, gemtuzumab ozogamicin, alemtuzumab, rituximab, and yttrium-90-ibritumomab tiuxetan), immuno-modulators (*e.g.,* thalidomide and lenalidomide), Bcr-Abl kinase inhibitors (*e.g.*, AP23464, AZD0530, CGP76030, PD180970, SKI-606, imatinib, BMS354825 (dasatinib), AMN107 (nilotinib), and VX-680), hormone agonists or antagonists, partial agonists or partial antagonists, kinase inhibitors, surgery, radiotherapy (*e.g.*, gamma-radiation, neutron bean radiotherapy, electron beam radiotherapy, proton therapy, brachytherapy, and systemic radioactive isotopes), endocrine therapy, biological response modifiers (*e.g.*, interferons, interleukins, and tumor necrosis factor), hyperthermia and cryotherapy, and agents to attenuate any adverse effects (*e.g.*, antiemetics).

[0048] As used herein, and unless otherwise specified, the terms "co-administration" and "in combination with" include the administration of two or more therapeutic agents simultaneously, concurrently or sequentially within no specific time limits unless otherwise indicated. In one embodiment, the agents are present in the cell or in the subject's body at the same time or exert their biological or therapeutic effect at the same time. In one embodiment,

the therapeutic agents are in the same composition or unit dosage form. In other embodiments, the therapeutic agents are in separate compositions or unit dosage forms. In certain embodiments, a first agent can be administered prior to (*e.g.*, 5 minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, or 12 weeks before), essentially concomitantly with, or subsequent to (*e.g.*, 5 minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, or 12 weeks after) the administration of a second therapeutic agent.

[0049] As used herein, and unless otherwise specified, the terms "composition," "formulation," and "dosage form" are intended to encompass products comprising the specified ingredient(s) (in the specified amounts, if indicated), as well as any product(s) which result, directly or indirectly, from combination of the specified ingredient(s) in the specified amount(s).

[0050] As used herein, and unless otherwise specified, the term "pharmaceutically acceptable carrier," "pharmaceutically acceptable excipient," "physiologically acceptable carrier," or "physiologically acceptable excipient" refers to a pharmaceutically-acceptable material, composition, or vehicle, such as a liquid or solid filler, diluent, excipient, solvent, or encapsulating material. In one embodiment, each component is "pharmaceutically acceptable" in the sense of being compatible with the other ingredients of a pharmaceutical formulation, and suitable for use in contact with the tissue or organ of humans and animals without excessive toxicity, irritation, allergic response, immunogenicity, or other problems or complications, commensurate with a reasonable benefit/risk ratio. In one embodiment, by "pharmaceutical" or "pharmaceutically acceptable" it is meant that any diluent(s), excipient(s) or carrier(s) in the composition, formulation, or dosage form are compatible with the other ingredient(s) and not deleterious to the recipient thereof. *See, e.g.,* Remington, *The Science and Practice of Pharmacy*, 21st Edition; Lippincott Williams & Wilkins: Philadelphia, PA, 2005; *Handbook of Pharmaceutical Excipients*, 5th Edition; Rowe *et al.*, ed., The Pharmaceutical Press and the American Pharmaceutical Association: 2005; and *Handbook of Pharmaceutical Additives*, 3rd Edition; Ash and Ash ed., Gower Publishing Company: 2007; *Pharmaceutical Preformulation and Formulation*, Gibson ed., CRC Press LLC: Boca Raton, FL, 2004.

[0051] As used herein, and unless otherwise specified, the term "hydrate" means a compound provided herein or a salt thereof, which further includes a stoichiometric or non-stoichiometric amount of water bound by non-covalent intermolecular forces.

[0052] As used herein, and unless otherwise specified, the term "solvate" means a solvate formed from the association of one or more solvent molecules to a compound provided herein. The term "solvate" includes hydrates (*e.g.*, hemihydrate, monohydrate, dihydrate, trihydrate, tetrahydrate, and the like).

[0053] As used herein, and unless otherwise specified, a compound described herein is intended to encompass all possible stereoisomers, unless a particular stereochemistry is specified. Where structural isomers of a compound are interconvertible *via* a low energy barrier, the compound may exist as a single tautomer or a mixture of tautomers. This can take the form of proton tautomerism; or so-called valence tautomerism in the compound, *e.g.*, that contain an aromatic moiety.

[0054] As used herein, and unless otherwise specified, in one embodiment, a compound described herein is intended to encompass isotopically enriched analogs. For example, one or more hydrogen position(s) in a compound may be enriched with deuterium and/or tritium. Other suitable isotopes that may be enriched at particular positions of a compound include, but are not limited, C-13, C-14, N-15, O-17, and/or O-18. In one embodiment, a compound described herein may be enriched at more than one position with isotopes, that are the same or different.

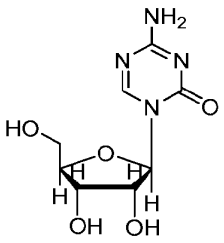
[0055] As used herein, and unless otherwise specified, a cytidine analog referred to herein is intended to encompass the free base of the cytidine analog, or a salt, solvate, hydrate, cocrystal, complex, prodrug, precursor, metabolite, and/or derivative thereof. In certain embodiments, a cytidine analog referred to herein encompasses the free base of the cytidine analog, or a salt, solvate, hydrate, cocrystal or complex thereof. In certain embodiments, a cytidine analog referred to herein encompasses the free base of the cytidine analog, or a pharmaceutically acceptable salt, solvate, or hydrate thereof.

5.2 Cytidine Analogs

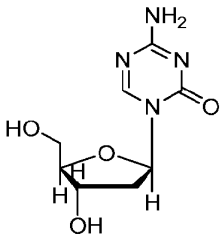
[0056] In one embodiment, the methods provided herein comprise administration or co-administration of one or more cytidine analogs. In certain embodiments, the cytidine analog is 5-azacytidine (azacitidine). In certain embodiments, the cytidine analog is 5-aza-2'-deoxycytidine (decitabine). In certain embodiments, the cytidine analog is 5-azacytidine (azacitidine) or 5-aza-2'-deoxycytidine (decitabine). In certain embodiments, the cytidine

analog is, for example: l-β-D-arabinofuranosylcytosine (Cytarabine or ara-C); pseudoiso-cytidine (psi ICR); 5-fluoro-2'-deoxycytidine (FCdR); 2'-deoxy-2',2'-difluorocytidine (Gemcitabine); 5-aza-2'-deoxy-2',2'-difluorocytidine; 5-aza-2'-deoxy-2'-fluorocytidine; l-β-D-ribofuranosyl-2(1*H*)-pyrimidinone (Zebularine); 2',3'-dideoxy-5-fluoro-3'-thiacytidine (Emtriva); 2'-cyclocytidine (Ancitabine); l-β-D-arabinofuranosyl-5-azacytosine (Fazarabine or ara-AC); 6-azacytidine (6-aza-CR); 5,6-dihydro-5-azacytidine (dH-aza-CR); N^4-pentyloxy-carbonyl-5'-deoxy-5-fluorocytidine (Capecitabine); N^4-octadecyl-cytarabine; or elaidic acid cytarabine. In certain embodiments, the cytidine analogs provided herein include any compound which is structurally related to cytidine or deoxycytidine and functionally mimics and/or antagonizes the action of cytidine or deoxycytidine.

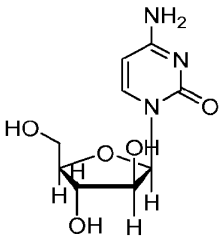
[0057] In certain embodiments, exemplary cytidine analogs have the structures provided below:



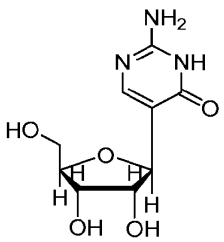
Azacitidine



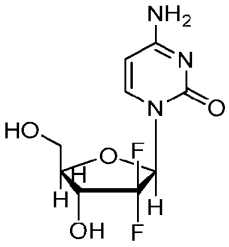
Decitabine



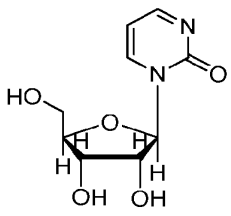
Cytarabine (Ara-C)



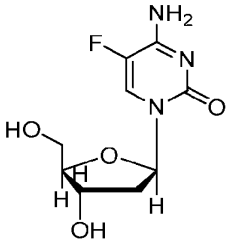
Pseudoisocytidine (psi ICR)



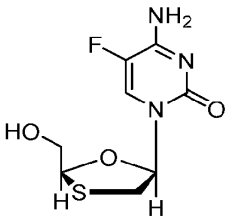
Gemcitabine



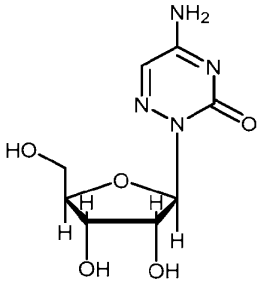
Zebularine



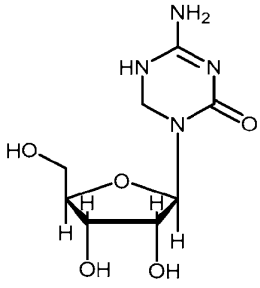
FCdR



Emtriva



6-Azacytidine



5-6-Dihydro-5-azacytidine

[0058] Certain embodiments herein provide salts, cocrystals, solvates (*e.g.*, hydrates), complexes, prodrugs, precursors, metabolites, and/or other derivatives of the cytidine analogs provided herein. For example, particular embodiments provide salts, cocrystals, solvates (*e.g.*, hydrates), complexes, precursors, metabolites, and/or other derivatives of 5-azacytidine. Certain embodiments herein provide salts, cocrystals, and/or solvates (*e.g.*, hydrates) of the cytidine analogs provided herein. Certain embodiments herein provide salts and/or solvates (*e.g.*, hydrates) of the cytidine analogs provided herein. Certain embodiments provide cytidine analogs that are not salts, cocrystals, solvates (*e.g.*, hydrates), or complexes of the cytidine analogs provided herein. For example, particular embodiments provide 5-azacytidine in a non-ionized, non-solvated (*e.g.*, anhydrous), non-complexed form. Certain embodiments herein provide a mixture of two or more cytidine analogs provided herein.

[0059] Cytidine analogs provided herein may be prepared using synthetic methods and procedures referenced herein or otherwise available in the literature. For example, particular methods for synthesizing 5-azacytidine are disclosed, *e.g.*, in U.S. Patent No. 7,038,038 and references discussed therein, each of which is incorporated herein by reference. Other cytidine analogs provided herein may be prepared, *e.g.*, using procedures known in the art, or may be purchased from a commercial source. In one embodiment, the cytidine analogs provided herein may be prepared in a particular solid form (*e.g.*, amorphous or crystalline form). *See, e.g.*, U.S. Patent Application No. 10/390,578, filed March 17, 2003 and U.S. Patent Application No. 10/390,530, filed March 17, 2003, both of which are incorporated herein by reference in their entireties.

[0060] In one embodiment, the compound used in the methods provided herein is a free base, or a pharmaceutically acceptable salt or solvate thereof. In one embodiment, the free base or the pharmaceutically acceptable salt or solvate is a solid. In another embodiment, the free base or the pharmaceutically acceptable salt or solvate is a solid in an amorphous form. In yet another embodiment, the free base or the pharmaceutically acceptable salt or solvate is a solid in a crystalline form. For example, particular embodiments provide 5-azacytidine in solid forms, which can be prepared, for example, according to the methods described in U.S. Patent Nos. 6,943,249, 6,887,855 and 7,078,518, and U.S. Patent Application Publication Nos. 2005/027675 and 2006/247189, each of which is incorporated by reference herein in their entireties. In other embodiments, 5-azacytidine in solid forms can be prepared using other methods known in the art.

[0061] In one embodiment, the compound used in the methods provided herein is a pharmaceutically acceptable salt of the cytidine analog, which includes, but is not limited to,

acetate, adipate, alginate, aspartate, benzoate, benzenesulfonate (besylate), bisulfate, butyrate, citrate, camphorate, camphorsulfonate, cyclopentanepropionate, digluconate, dodecylsulfate, 1,2-ethanedisulfonate (edisylate), ethanesulfonate (esylate), formate, fumarate, glucoheptanoate, glycerophosphate, glycolate, hemisulfate, heptanoate, hexanoate, hydrochloride, hydrobromide, hydroiodide, 2-hydroxyethanesulfonate, lactate, maleate, malonate, methanesulfonate (mesylate), 2-naphthalenesulfonate (napsylate), nicotinate, nitrate, oxalate, palmoate, pectinate, persulfate, 3-phenylpropionate, phosphate, picrate, pivalate, propionate, salicylate, succinate, sulfate, tartrate, thiocyanate, tosylate, or undecanoate salts.

5.3 Pharmaceutical Compositions

[0062] In one embodiment, provided herein are pharmaceutical compositions, which comprise one or more cytidine analogs, or a pharmaceutically acceptable salt or solvate thereof, as an active ingredient, in combination with one or more pharmaceutically acceptable carrier. In one embodiment, the pharmaceutical composition comprises at least one nonrelease controlling excipient or carrier. In one embodiment, the pharmaceutical composition comprises at least one release controlling and at least one nonrelease controlling excipients or carriers.

[0063] In certain embodiments, the cytidine analog used in the pharmaceutical compositions provided herein is in a solid form. Suitable solid forms include, but are not limited to, solid forms comprising the free base of the cytidine analog, and solid forms comprising salts of the cytidine analog. In certain embodiments, solid forms provided herein include polymorphs, solvates (including hydrates), and cocrystals comprising the cytidine analog and/or salts thereof. In certain embodiments, the solid form is a crystal form of the cytidine analog, or a pharmaceutically acceptable salt or solvate thereof.

[0064] In one embodiment, the pharmaceutical compositions provided herein may be formulated in various dosage forms for oral, parenteral, and topical administration. The pharmaceutical compositions may also be formulated as modified release dosage forms, including delayed-, extended-, prolonged-, sustained-, pulsed-, controlled-, accelerated- and fast-, targeted-, programmed-release, and gastric retention dosage forms. These dosage forms can be prepared according to conventional methods and techniques known to those skilled in the art (*see, e.g.,* Remington, *The Science and Practice of Pharmacy*, 21st Edition; Lippincott Williams & Wilkins: Philadelphia, PA, 2005; *Modified-Release Drug Delivery Technology*,

Rathbone *et al.*, eds., Drugs and the Pharmaceutical Science, Marcel Dekker, Inc.: New York, NY, 2003; Vol. 126).

[0065] In one embodiment, the pharmaceutical compositions are provided in a dosage form for oral administration. In another embodiment, the pharmaceutical compositions are provided in a dosage form for parenteral administration. In yet another embodiment, the pharmaceutical compositions are provided in a dosage form for topical administration.

[0066] In one embodiment, the pharmaceutical compositions provided herein may be provided in a unit-dosage form or multiple-dosage form. A unit-dosage form, as used herein, refers to a physically discrete unit suitable for administration to human and animal subjects, and packaged individually as is known in the art. Each unit-dose contains a predetermined quantity of the active ingredient(s) sufficient to produce the desired therapeutic effect, in association with the required pharmaceutical carriers or excipients. Examples of a unit-dosage form include an ampoule, syringe, and individually packaged tablet and capsule. A unit-dosage form may be administered in fractions or multiples thereof. A multiple-dosage form is a plurality of identical unit-dosage forms packaged in a single container to be administered in segregated unit-dosage form. Examples of a multiple-dosage form include a vial, bottle of tablets or capsules, or bottle of pints or gallons.

[0067] In one embodiment, the pharmaceutical compositions provided herein may be administered at once or multiple times at intervals of time. It is understood that the precise dosage and duration of treatment may vary with the age, weight, and condition of the patient being treated, and may be determined empirically using known testing protocols or by extrapolation from *in vivo* or *in vitro* test or diagnostic data. It is further understood that for any particular individual, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the formulations.

5.3.1 Oral Administration

[0068] In one embodiment, the pharmaceutical compositions provided herein may be provided in solid, semisolid, or liquid dosage forms for oral administration. As used herein, oral administration also includes buccal, lingual, and sublingual administration. Suitable oral dosage forms include, but are not limited to, tablets, capsules, pills, troches, lozenges, pastilles, cachets, pellets, medicated chewing gum, granules, bulk powders, effervescent or non-effervescent powders or granules, solutions, emulsions, suspensions, solutions, wafers, sprinkles, elixirs, and syrups. In addition to the active ingredient(s), the pharmaceutical

compositions may contain one or more pharmaceutically acceptable carriers or excipients, including, but not limited to, binders, fillers, diluents, disintegrants, wetting agents, lubricants, glidants, coloring agents, dye-migration inhibitors, sweetening agents, and flavoring agents.

[0069] In one embodiment, binders or granulators impart cohesiveness to a tablet to ensure the tablet remaining intact after compression. Suitable binders or granulators include, but are not limited to, starches, such as corn starch, potato starch, and pre-gelatinized starch (*e.g.*, STARCH 1500); gelatin; sugars, such as sucrose, glucose, dextrose, molasses, and lactose; natural and synthetic gums, such as acacia, alginic acid, alginates, extract of Irish moss, panwar gum, ghatti gum, mucilage of isabgol husks, carboxymethylcellulose, methylcellulose, polyvinylpyrrolidone (PVP), Veegum, larch arabogalactan, powdered tragacanth, and guar gum; celluloses, such as ethyl cellulose, cellulose acetate, carboxymethyl cellulose calcium, sodium carboxymethyl cellulose, methyl cellulose, hydroxyethylcellulose (HEC), hydroxypropylcellulose (HPC), hydroxypropyl methyl cellulose (HPMC); microcrystalline celluloses, such as AVICEL-PH-101, AVICEL-PH-103, AVICEL RC-581, AVICEL-PH-105 (FMC Corp., Marcus Hook, PA); and mixtures thereof. Suitable fillers include, but are not limited to, talc, calcium carbonate, microcrystalline cellulose, powdered cellulose, dextrates, kaolin, mannitol, silicic acid, sorbitol, starch, pre-gelatinized starch, and mixtures thereof. The binder or filler may be present from about 50 to about 99% by weight in the pharmaceutical compositions provided herein.

[0070] In one embodiment, suitable diluents include, but are not limited to, dicalcium phosphate, calcium sulfate, lactose, sorbitol, sucrose, inositol, cellulose, kaolin, mannitol, sodium chloride, dry starch, and powdered sugar. Certain diluents, such as mannitol, lactose, sorbitol, sucrose, and inositol, when present in sufficient quantity, can impart properties to some compressed tablets that permit disintegration in the mouth by chewing. Such compressed tablets can be used as chewable tablets.

[0071] In one embodiment, suitable disintegrants include, but are not limited to, agar; bentonite; celluloses, such as methylcellulose and carboxymethylcellulose; wood products; natural sponge; cation-exchange resins; alginic acid; gums, such as guar gum and Veegum HV; citrus pulp; cross-linked celluloses, such as croscarmellose; cross-linked polymers, such as crospovidone; cross-linked starches; calcium carbonate; microcrystalline cellulose, such as sodium starch glycolate; polacrilin potassium; starches, such as corn starch, potato starch, tapioca starch, and pre-gelatinized starch; clays; aligns; and mixtures thereof. The amount of a disintegrant in the pharmaceutical compositions provided herein varies upon the type of

formulation, and is readily discernible to those of ordinary skill in the art. The pharmaceutical compositions provided herein may contain from about 0.5 to about 15% or from about 1 to about 5% by weight of a disintegrant.

[0072] In one embodiment, suitable lubricants include, but are not limited to, calcium stearate; magnesium stearate; mineral oil; light mineral oil; glycerin; sorbitol; mannitol; glycols, such as glycerol behenate and polyethylene glycol (PEG) (*e.g.*, PEG400 and PEG6000); stearic acid; sodium lauryl sulfate; talc; hydrogenated vegetable oil, including peanut oil, cottonseed oil, sunflower oil, sesame oil, olive oil, corn oil, and soybean oil; zinc stearate; ethyl oleate; ethyl laureate; agar; starch; lycopodium; silica (silicone dioxide) or silica gels, such as AEROSIL® 200 (W.R. Grace Co., Baltimore, MD) and CAB-O-SIL® (Cabot Co. of Boston, MA); and mixtures thereof. The pharmaceutical compositions provided herein may contain about 0.1 to about 5% by weight of a lubricant.

[0073] In one embodiment, suitable glidants include colloidal silicon dioxide, CAB-O-SIL® (Cabot Co. of Boston, MA), and asbestos-free talc. Coloring agents include any of the approved, certified, water soluble FD&C dyes, and water insoluble FD&C dyes suspended on alumina hydrate, and color lakes and mixtures thereof. A color lake is the combination by adsorption of a water-soluble dye to a hydrous oxide of a heavy metal, resulting in an insoluble form of the dye. Flavoring agents include natural flavors extracted from plants, such as fruits, and synthetic blends of compounds which produce a pleasant taste sensation, such as peppermint and methyl salicylate. Sweetening agents include sucrose, lactose, mannitol, syrups, glycerin, and artificial sweeteners, such as saccharin and aspartame. Suitable emulsifying agents include gelatin, acacia, tragacanth, bentonite, and surfactants, such as polyoxyethylene sorbitan monooleate (*e.g.*, TWEEN® 20), poloxamers (*e.g.*, PLURONIC® F68), polyoxyethylene sorbitan monooleate 80 (*e.g.*, TWEEN® 80), and triethanolamine oleate. Suspending and dispersing agents include sodium carboxymethylcellulose, pectin, tragacanth, Veegum, acacia, sodium carbomethylcellulose, hydroxypropyl methylcellulose, polyvinylpyrrolidone, and lauroyl polyoxylglycerides (*e.g.*, GELUCIRE® 44/14). Preservatives include glycerin, methyl and propylparaben, benzoic add, sodium benzoate and alcohol. Wetting agents include propylene glycol monostearate, sorbitan monooleate, diethylene glycol monolaurate, and polyoxyethylene lauryl ether. Solvents include glycerin, sorbitol, ethyl alcohol, and syrup. Examples of non-aqueous liquids utilized in emulsions include mineral oil and cottonseed oil. Organic acids include citric and tartaric acid. Sources of carbon dioxide include sodium bicarbonate and sodium carbonate.

[0074] In one embodiment, suitable complexing agents include, but are not limited to, cyclodextrins, including α-cyclodextrin, β-cyclodextrin, hydroxypropyl-β-cyclodextrin, sulfobutylether-β-cyclodextrin, and sulfobutylether 7-β-cyclodextrin (CAPTISOL®, CyDex, Lenexa, KS).

[0075] It should be understood that many carriers and excipients may serve several functions, even within the same formulation.

[0076] In one embodiment, the pharmaceutical compositions provided herein may be provided as compressed tablets, tablet triturates, chewable lozenges, rapidly dissolving tablets, multiple compressed tablets, or enteric-coating tablets, sugar-coated, or film-coated tablets. In one embodiment, enteric-coated tablets are compressed tablets coated with substances that resist the action of stomach acid but dissolve or disintegrate in the intestine, thus protecting the active ingredients from the acidic environment of the stomach. Enteric-coatings include, but are not limited to, fatty acids, fats, phenyl salicylate, waxes, shellac, ammoniated shellac, and cellulose acetate phthalates. Sugar-coated tablets are compressed tablets surrounded by a sugar coating, which may be beneficial in covering up objectionable tastes or odors and in protecting the tablets from oxidation. Film-coated tablets are compressed tablets that are covered with a thin layer or film of a water-soluble material. Film coatings include, but are not limited to, hydroxyethylcellulose, sodium carboxymethylcellulose, polyethylene glycol 4000, and cellulose acetate phthalate. In one embodiment, film coating imparts the same general characteristics as sugar coating. Multiple compressed tablets are compressed tablets made by more than one compression cycle, including layered tablets, and press-coated or dry-coated tablets.

[0077] In one embodiment, the tablet dosage forms may be prepared from the active ingredient in powdered, crystalline, or granular forms, alone or in combination with one or more carriers or excipients described herein, including binders, disintegrants, controlled-release polymers, lubricants, diluents, and/or colorants. Flavoring and sweetening agents are especially useful in the formation of chewable tablets and lozenges.

[0078] In one embodiment, the pharmaceutical compositions provided herein may be provided as soft or hard capsules, which can be made from gelatin, methylcellulose, starch, or calcium alginate. The hard gelatin capsule, also known as the dry-filled capsule (DFC), consists of two sections, one slipping over the other, thus completely enclosing the active ingredient. The soft elastic capsule (SEC) is a soft, globular shell, such as a gelatin shell, which is plasticized by the addition of glycerin, sorbitol, or a similar polyol. The soft gelatin

shells may contain a preservative to prevent the growth of microorganisms. Suitable preservatives are those as described herein, including methyl- and propyl-parabens, and sorbic acid. The liquid, semisolid, and solid dosage forms provided herein may be encapsulated in a capsule. Suitable liquid and semisolid dosage forms include solutions and suspensions in propylene carbonate, vegetable oils, or triglycerides. Capsules containing such solutions can be prepared as described in U.S. Patent Nos. 4,328,245; 4,409,239; and 4,410,545. The capsules may also be coated as known by those of skill in the art in order to modify or sustain dissolution of the active ingredient.

[0079] In one embodiment, the pharmaceutical compositions provided herein may be provided in liquid and semisolid dosage forms, including emulsions, solutions, suspensions, elixirs, and syrups. An emulsion is a two-phase system, in which one liquid is dispersed in the form of small globules throughout another liquid, which can be oil-in-water or water-in-oil. Emulsions may include a pharmaceutically acceptable non-aqueous liquid or solvent, emulsifying agent, and preservative. Suspensions may include a pharmaceutically acceptable suspending agent and preservative. Aqueous alcoholic solutions may include a pharmaceutically acceptable acetal, such as a di(lower alkyl) acetal of a lower alkyl aldehyde, *e.g.*, acetaldehyde diethyl acetal; and a water-miscible solvent having one or more hydroxyl groups, such as propylene glycol and ethanol. Elixirs are clear, sweetened, and hydroalcoholic solutions. Syrups are concentrated aqueous solutions of a sugar, for example, sucrose, and may also contain a preservative. For a liquid dosage form, for example, a solution in a polyethylene glycol may be diluted with a sufficient quantity of a pharmaceutically acceptable liquid carrier, *e.g.*, water, to be measured conveniently for administration.

[0080] In one embodiment, other useful liquid and semisolid dosage forms include, but are not limited to, those containing the active ingredient(s) provided herein, and a dialkylated mono- or poly-alkylene glycol, including, 1,2-dimethoxymethane, diglyme, triglyme, tetraglyme, polyethylene glycol-350-dimethyl ether, polyethylene glycol-550-dimethyl ether, polyethylene glycol-750-dimethyl ether, wherein 350, 550, and 750 refer to the approximate average molecular weight of the polyethylene glycol. These formulations may further comprise one or more antioxidants, such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate, vitamin E, hydroquinone, hydroxycoumarins, ethanolamine, lecithin, cephalin, ascorbic acid, malic acid, sorbitol, phosphoric acid, bisulfite, sodium metabisulfite, thiodipropionic acid and its esters, and dithiocarbamates.

[0081] In one embodiment, the pharmaceutical compositions provided herein may be provided as non-effervescent or effervescent, granules and powders, to be reconstituted into a liquid dosage form. Pharmaceutically acceptable carriers and excipients used in the non-effervescent granules or powders may include diluents, sweeteners, and wetting agents. Pharmaceutically acceptable carriers and excipients used in the effervescent granules or powders may include organic acids and a source of carbon dioxide.

[0082] Coloring and flavoring agents can be used in all of the above dosage forms.

[0083] In one embodiment, the pharmaceutical compositions provided herein may be formulated as immediate or modified release dosage forms, including delayed-, sustained, pulsed-, controlled, targeted-, and programmed-release forms.

[0084] In one embodiment, the pharmaceutical compositions provided herein may be co-formulated with other active ingredients which do not impair the desired therapeutic action, or with substances that supplement the desired action.

[0085] In one embodiment, active ingredients provided herein can be administered by controlled release means or by delivery devices that are well known to those of ordinary skill in the art. Examples include, but are not limited to, those described in U.S. Patent Nos.: 3,845,770; 3,916,899; 3,536,809; 3,598,123; and 4,008,719, 5,674,533, 5,059,595, 5,591,767, 5,120,548, 5,073,543, 5,639,476, 5,354,556, and 5,733,566, each of which is incorporated herein by reference. Such dosage forms can be used to provide slow or controlled-release of one or more active ingredients using, for example, hydropropylmethyl cellulose, other polymer matrices, gels, permeable membranes, osmotic systems, multilayer coatings, microparticles, liposomes, microspheres, or a combination thereof to provide the desired release profile in varying proportions. Suitable controlled-release formulations known to those of ordinary skill in the art, including those described herein, can be readily selected for use with the active agents provided herein. In one embodiment, provided are single unit dosage forms suitable for oral administration such as, but not limited to, tablets, capsules, gelcaps, and caplets that are adapted for controlled-release.

[0086] In one embodiment, controlled-release pharmaceutical products improve drug therapy over that achieved by their non-controlled counterparts. In another embodiment, the use of a controlled-release preparation in medical treatment is characterized by a minimum of drug substance being employed to cure or control the condition in a minimum amount of time. Advantages of controlled-release formulations include extended activity of the drug, reduced dosage frequency, and increased patient compliance. In addition, controlled-release

formulations can be used to affect the time of onset of action or other characteristics, such as blood levels of the drug, and can thus affect the occurrence of side (*e.g.*, adverse) effects.

[0087] In another embodiment, the controlled-release formulations are designed to initially release an amount of drug (active ingredient) that promptly produces the desired therapeutic or prophylactic effect, and gradually and continually release of other amounts of drug to maintain this level of therapeutic or prophylactic effect over an extended period of time. In one embodiment, in order to maintain a constant level of drug in the body, the drug can be released from the dosage form at a rate that will replace the amount of drug being metabolized and excreted from the body. Controlled-release of an active ingredient can be stimulated by various conditions including, but not limited to, pH, temperature, enzymes, water, or other physiological conditions or compounds.

[0088] In one embodiment, provided herein is an oral formulation of a cytidine analog. *See, e.g.*, U.S. Patent Application No. 12/466,213, filed May 14, 2009 and U.S. Patent Application No. 11/849,958, filed September 4, 2007, both of which are incorporated herein by reference in their entireties.

5.3.2 Parenteral Administration

[0089] In one embodiment, the pharmaceutical compositions provided herein may be administered parenterally by injection, infusion, or implantation, for local or systemic administration. Parenteral administration, as used herein, include intravenous, intraarterial, intraperitoneal, intrathecal, intraventricular, intraurethral, intrasternal, intracranial, intramuscular, intrasynovial, and subcutaneous administration.

[0090] In one embodiment, the pharmaceutical compositions provided herein may be formulated in any dosage forms that are suitable for parenteral administration, including solutions, suspensions, emulsions, micelles, liposomes, microspheres, nanosystems, and solid forms suitable for solutions or suspensions in liquid prior to injection. Such dosage forms can be prepared according to conventional methods known to those skilled in the art of pharmaceutical science (*see, e.g.,* Remington, *The Science and Practice of Pharmacy*, *supra*).

[0091] In one embodiment, the pharmaceutical compositions intended for parenteral administration may include one or more pharmaceutically acceptable carriers and excipients, including, but not limited to, aqueous vehicles, water-miscible vehicles, non-aqueous vehicles, antimicrobial agents or preservatives against the growth of microorganisms, stabilizers, solubility enhancers, isotonic agents, buffering agents, antioxidants, local anesthetics, suspending and dispersing agents, wetting or emulsifying agents, complexing

agents, sequestering or chelating agents, cryoprotectants, lyoprotectants, thickening agents, pH adjusting agents, and inert gases.

[0092] In one embodiment, suitable aqueous vehicles include, but are not limited to, water, saline, physiological saline or phosphate buffered saline (PBS), sodium chloride injection, Ringers injection, isotonic dextrose injection, sterile water injection, dextrose and lactated Ringers injection. Non-aqueous vehicles include, but are not limited to, fixed oils of vegetable origin, castor oil, corn oil, cottonseed oil, olive oil, peanut oil, peppermint oil, safflower oil, sesame oil, soybean oil, hydrogenated vegetable oils, hydrogenated soybean oil, and medium-chain triglycerides of coconut oil, and palm seed oil. Water-miscible vehicles include, but are not limited to, ethanol, 1,3-butanediol, liquid polyethylene glycol (*e.g.*, polyethylene glycol 300 and polyethylene glycol 400), propylene glycol, glycerin, *N*-methyl-2-pyrrolidone, *N,N*-dimethylacetamide, and dimethyl sulfoxide.

[0093] In one embodiment, suitable antimicrobial agents or preservatives include, but are not limited to, phenols, cresols, mercurials, benzyl alcohol, chlorobutanol, methyl and propyl p-hydroxybenzoates, thimerosal, benzalkonium chloride (*e.g.*, benzethonium chloride), methyl- and propyl-parabens, and sorbic acid. Suitable isotonic agents include, but are not limited to, sodium chloride, glycerin, and dextrose. Suitable buffering agents include, but are not limited to, phosphate and citrate. Suitable antioxidants are those as described herein, including bisulfite and sodium metabisulfite. Suitable local anesthetics include, but are not limited to, procaine hydrochloride. Suitable suspending and dispersing agents are those as described herein, including sodium carboxymethylcelluose, hydroxypropyl methylcellulose, and polyvinylpyrrolidone. Suitable emulsifying agents include those described herein, including polyoxyethylene sorbitan monolaurate, polyoxyethylene sorbitan monooleate 80, and triethanolamine oleate. Suitable sequestering or chelating agents include, but are not limited to EDTA. Suitable pH adjusting agents include, but are not limited to, sodium hydroxide, hydrochloric acid, citric acid, and lactic acid. Suitable complexing agents include, but are not limited to, cyclodextrins, including α-cyclodextrin, β-cyclodextrin, hydroxypropyl-β-cyclodextrin, sulfobutylether-β-cyclodextrin, and sulfobutylether 7-β-cyclodextrin (CAPTISOL®, CyDex, Lenexa, KS).

[0094] In one embodiment, the pharmaceutical compositions provided herein may be formulated for single or multiple dosage administration. The single dosage formulations are packaged in an ampoule, a vial, or a syringe. The multiple dosage parenteral formulations

may contain an antimicrobial agent at bacteriostatic or fungistatic concentrations. All parenteral formulations must be sterile, as known and practiced in the art.

[0095] In one embodiment, the pharmaceutical compositions are provided as ready-to-use sterile solutions. In another embodiment, the pharmaceutical compositions are provided as sterile dry soluble products, including lyophilized powders and hypodermic tablets, to be reconstituted with a vehicle prior to use. In yet another embodiment, the pharmaceutical compositions are provided as ready-to-use sterile suspensions. In yet another embodiment, the pharmaceutical compositions are provided as sterile dry insoluble products to be reconstituted with a vehicle prior to use. In still another embodiment, the pharmaceutical compositions are provided as ready-to-use sterile emulsions.

[0096] In one embodiment, the pharmaceutical compositions provided herein may be formulated as immediate or modified release dosage forms, including delayed-, sustained, pulsed-, controlled, targeted-, and programmed-release forms.

[0097] In one embodiment, the pharmaceutical compositions may be formulated as a suspension, solid, semi-solid, or thixotropic liquid, for administration as an implanted depot. In one embodiment, the pharmaceutical compositions provided herein are dispersed in a solid inner matrix, which is surrounded by an outer polymeric membrane that is insoluble in body fluids but allows the active ingredient in the pharmaceutical compositions diffuse through.

[0098] In one embodiment, suitable inner matrixes include polymethylmethacrylate, polybutyl-methacrylate, plasticized or unplasticized polyvinylchloride, plasticized nylon, plasticized polyethylene terephthalate, natural rubber, polyisoprene, polyisobutylene, polybutadiene, polyethylene, ethylene-vinyl acetate copolymers, silicone rubbers, polydimethylsiloxanes, silicone carbonate copolymers, hydrophilic polymers, such as hydrogels of esters of acrylic and methacrylic acid, collagen, cross-linked polyvinyl alcohol, and cross-linked partially hydrolyzed polyvinyl acetate.

[0099] In one embodiment, suitable outer polymeric membranes include polyethylene, polypropylene, ethylene/propylene copolymers, ethylene/ethyl acrylate copolymers, ethylene/vinyl acetate copolymers, silicone rubbers, polydimethyl siloxanes, neoprene rubber, chlorinated polyethylene, polyvinylchloride, vinyl chloride copolymers with vinyl acetate, vinylidene chloride, ethylene and propylene, ionomer polyethylene terephthalate, butyl rubber epichlorohydrin rubbers, ethylene/vinyl alcohol copolymer, ethylene/vinyloxyethanol copolymer, and ethylene/vinyl acetate/vinyl alcohol terpolymer.

[00100] In specific embodiments, the pharmaceutical composition provided herein comprise 5-azacytidine and mannitol. In specific embodiments, the pharmaceutical

composition provided herein is a lyophilized powder. In specific embodiments, the pharmaceutical composition provided herein is a lyophilized powder comprising 5-azacytidine and mannitol. In specific embodiments, the pharmaceutical composition provided herein comprises 5-azacytidine and mannitol with a relative weight ratio of about 1:1 w/w. In specific embodiments, the pharmaceutical composition provided herein comprises about 100 mg of 5-azacytidine. In specific embodiments, the pharmaceutical composition provided herein comprises about 100 mg of 5-azacytidine and about 100 mg of mannitol.

5.3.3 Topical Administration

[00101] In one embodiment, the pharmaceutical compositions provided herein may be administered rectally, urethrally, vaginally, or perivaginally in the forms of suppositories, pessaries, bougies, poultices or cataplasm, pastes, powders, dressings, creams, plasters, contraceptives, ointments, solutions, emulsions, suspensions, tampons, gels, foams, sprays, or enemas. These dosage forms can be manufactured using conventional processes as described in, *e.g.*, Remington, *The Science and Practice of Pharmacy*, *supra*.

[00102] In one embodiment, rectal, urethral, and vaginal suppositories are solid bodies for insertion into body orifices, which are solid at ordinary temperatures but melt or soften at body temperature to release the active ingredient(s) inside the orifices. Pharmaceutically acceptable carriers utilized in rectal and vaginal suppositories include bases or vehicles, such as stiffening agents, which produce a melting point in the proximity of body temperature, when formulated with the pharmaceutical compositions provided herein; and antioxidants as described herein, including bisulfite and sodium metabisulfite. Suitable vehicles include, but are not limited to, cocoa butter (theobroma oil), glycerin-gelatin, carbowax (polyoxyethylene glycol), spermaceti, paraffin, white and yellow wax, and appropriate mixtures of mono-, di- and triglycerides of fatty acids, hydrogels, such as polyvinyl alcohol, hydroxyethyl methacrylate, polyacrylic acid; glycerinated gelatin. Combinations of the various vehicles may be used. Rectal and vaginal suppositories may be prepared by the compressed method or molding. The typical weight of a rectal and vaginal suppository is about 2 to about 3 g.

[00103] In one embodiment, the pharmaceutical compositions provided herein may be administered intranasally or by inhalation to the respiratory tract. The pharmaceutical compositions may be provided in the form of an aerosol or solution for delivery using a pressurized container, pump, spray, atomizer, such as an atomizer using electrohydrodynamics to produce a fine mist, or nebulizer, alone or in combination with a

suitable propellant, such as 1,1,1,2-tetrafluoroethane or 1,1,1,2,3,3,3-heptafluoropropane. The pharmaceutical compositions may also be provided as a dry powder for insufflation, alone or in combination with an inert carrier such as lactose or phospholipids; and nasal drops. For intranasal use, the powder may comprise a bioadhesive agent, including chitosan or cyclodextrin.

[00104] In one embodiment, solutions or suspensions for use in a pressurized container, pump, spray, atomizer, or nebulizer may be formulated to contain ethanol, aqueous ethanol, or a suitable alternative agent for dispersing, solubilizing, or extending release of the active ingredient provided herein, a propellant as solvent; and/or a surfactant, such as sorbitan trioleate, oleic acid, or an oligolactic acid.

[00105] In one embodiment, the pharmaceutical compositions provided herein may be micronized to a size suitable for delivery by inhalation, such as about 50 micrometers or less, or about 10 micrometers or less. Particles of such sizes may be prepared using a comminuting method known to those skilled in the art, such as spiral jet milling, fluid bed jet milling, supercritical fluid processing to form nanoparticles, high pressure homogenization, or spray drying.

[00106] In one embodiment, capsules, blisters and cartridges for use in an inhaler or insufflator may be formulated to contain a powder mix of the pharmaceutical compositions provided herein; a suitable powder base, such as lactose or starch; and a performance modifier, such as *L*-leucine, mannitol, or magnesium stearate. The lactose may be anhydrous or in the form of the monohydrate. Other suitable excipients or carriers include dextran, glucose, maltose, sorbitol, xylitol, fructose, sucrose, and trehalose. The pharmaceutical compositions provided herein for inhaled/intranasal administration may further comprise a suitable flavor, such as menthol and levomenthol, or sweeteners, such as saccharin or saccharin sodium.

[00107] In one embodiment, the pharmaceutical compositions provided herein for topical administration may be formulated to be immediate release or modified release, including delayed-, sustained-, pulsed-, controlled-, targeted, and programmed release.

5.3.4 Kits

[00108] In one embodiment, active ingredients provided herein are not administered to a patient at the same time or by the same route of administration. In another embodiment, provided are kits which can simplify the administration of appropriate amounts of active ingredients.

[00109] In one embodiment, a kit comprises a dosage form of a compound provided herein. Kits can further comprise one or more second active ingredients as described herein, or a pharmacologically active mutant or derivative thereof, or a combination thereof.

[00110] In other embodiments, kits can further comprise devices that are used to administer the active ingredients. Examples of such devices include, but are not limited to, syringes, drip bags, patches, and inhalers.

[00111] In one embodiment, kits can further comprise cells or blood for transplantation as well as pharmaceutically acceptable vehicles that can be used to administer one or more active ingredients. For example, if an active ingredient is provided in a solid form that must be reconstituted for parenteral administration, the kit can comprise a sealed container of a suitable vehicle in which the active ingredient can be dissolved to form a particulate-free sterile solution that is suitable for parenteral administration. Examples of pharmaceutically acceptable vehicles include, but are not limited to: Water for Injection USP; aqueous vehicles such as, but not limited to, Sodium Chloride Injection, Ringer's Injection, Dextrose Injection, Dextrose and Sodium Chloride Injection, and Lactated Ringer's Injection; water-miscible vehicles such as, but not limited to, ethyl alcohol, polyethylene glycol, and polypropylene glycol; and non-aqueous vehicles such as, but not limited to, corn oil, cottonseed oil, peanut oil, sesame oil, ethyl oleate, isopropyl myristate, and benzyl benzoate.

5.4 Method of Use

[00112] Without being limited to a particular theory, in one embodiment, 5-azacytidine is approved for treatment of patients with higher-risk MDS. In certain clinical studies, treatment with 5-azacytidine increases median overall survival by about 9.5 months in patients with higher-risk MDS in comparison to conventional care regimens (CCR). In these studies, the median overall survival for 5-azacytidine-treated patients having higher-risk MDS is about two years.

[00113] Without being limited to a particular theory, in one embodiment, development of biomarkers predictive of maximal clinical benefit with 5-azacytidine would allow identification of those patients particularly suited for 5-azacytidine therapy. Thus, in one embodiment, provided herein are biomarkers that could be used, for example, in the management of therapeutic choices for patients with MDS. In other embodiments, provided herein is a method of using a biomarker provided herein in selecting cancer patients for a particular therapy, *e.g.*, 5-azacytidine therapy for a particular cancer, to derive maximal clinical benefits from that therapy.

[00114] Without being limited to a particular theory, DNA methylation plays an essential role in the regulation of gene expression. *See, e.g.*, Esteller M., *New England Journal of Medicine*, 2008, 358:1148–59. For example, CpG islands are generally unmethylated in normal cells, and demethylated state allows gene transcription. Methylation of CpG islands may be associated with gene silencing, including imprinted genes and X-chromosome inactivation. On the other hand, CpG sites outside CpG islands are generally methylated in normal cells, and methylation helps prevent mutations and genomic instability, and helps prevent recombination and activation of transposable elements. Large alternations in DNA methylation patterns are observed in most human cancers. CpG islands may become hypermethylated, while CpG sites outside of CpG islands may become hypomethylated. Thus, DNA methylation may be used as biomarkers for cancers or for MDS.

[00115] In one embodiment, provided herein are predictive biomarkers for assessing potential clinical benefit of a cancer therapy. In one embodiment, provided herein are predictive biomarkers for assessing potential clinical benefit of an MDS therapy. In one embodiment, provided herein are predictive biomarkers for assessing potential clinical benefit of 5-azacytidine therapy. In one embodiment, provided herein are methods of using a predictive biomarker provided herein (*e.g.*, baseline patterns or levels of DNA methylation in pre-treatment bio-samples from patients). In one embodiment, the clinical benefit includes, but is not limited to, prolonged survival, delayed progression to AML, and/or other beneficial clinical responses. In one embodiment, the biomarker provided herein is nucleic acid methylation of one or more particular gene(s) or one or more particular locus/loci. In one embodiment, the biomarker provided herein is DNA methylation of one or more particular gene(s) or one or more particular locus/loci. In one embodiment, the predictive or response biomarkers provided herein include, but are not limited to, one or more of the following: DNA methylation, RNA methylation, previous LD AraC treatment, bone marrow blasts, abnormal karyotype, performance status, intermediate and poor risk cytogenetics, presence of circulating blasts, and/or RBC transfusion dependency. In one embodiment, the predictive or response biomarkers provided herein can be used to assess or predict response rate, overall survival, or other clinical responses.

[00116] In some embodiments, high baseline methylation of specific genes, such as, *e.g.*, *CDKN2B* (*p15*), *SOCS1*, *CDH1*, *TP73*, and/or *CTNNA1*, may be indicative of poor overall survival as compared to subjects with lower methylation levels.

[00117] In one embodiment, methylation pattern of specific group of genes may be indicative of better or worse overall survival after a particular therapeutic treatment. In one

embodiment, provided herein is a methylation pattern of particular genes, selected from the group consisting of: *ABHD14A*, *ABO*, *ADAMTS18*, *ADRA2B*, *ADRB3*, *AIRE*, *AKAP12*, *ALOX15B*, *ALS2CR11*, *AMT*, *ANKRD33*, *APC2*, *AVP*, *BHMT*, *C18orf22*, *C19orf30*, *C1orf172*, *C1orf87*, *C3orf15*, *C1QTNF6*, *C22orf27*, *C7orf16*, *C7orf41*, *CBX7*, *CCDC19*, *CCDC81*, *CD164L2*, *CDH1*, *CDKN2B*, *CHAD*, *CHRNG*, *CIDEB*, *CKMT1B*, *CKMT2*, *CLCN6*, *CLDN6*, *CLDN9*, *CNTN4*, *CPT1B*, *CRHBP*, *CXCL5*, *CYP26C1*, *CYP2E1*, *DES*, *DPYS*, *DYDC1*, *EGFL7*, *ELMO3*, *ENTPD2*, *ENTPD3*, *ESR1*, *EYA4*, *F2RL2*, *FAM57B*, *FBLN1*, *FBXO2*, *FKBP1B*, *FLJ44881*, *FLVCR2*, *FREQ*, *FZD9*, *GAB1*, *GAS2L2*, *GATA4*, *GBGT1*, *GDF5*, *GHSR*, *GNAS*, *GNMT*, *GNPNAT1*, *GP1BA*, *GPR25*, *GRM6*, *GSTM5*, *HCN4*, *HIST1H1A*, *HOXD4*, *HSPA2*, *HTATIP2*, *HTR7*, *HYDIN*, *IGDCC3*, *ILDR1*, *IRF6*, *KAZALD1*, *KCNA6*, *KCNK3*, *KCNQ1*, *KIAA0427*, *KIR3DX1*, *KRT25*, *KRT7*, *KRT72*, *LAD1*, *LAMA4*, *LAMC2*, *LGTN*, *LRRC17*, *LTF*, *MBD3L1*, *MEGF10*, *MICAL1*, *MRPL28*, *MTMR9*, *MTNR1B*, *NALCN*, *NCAN*, *NCOR2*, *NDRG2*, *NDUFAF3*, *NEUROG1*, *NGB*, *NPFFR2*, *NPM2*, *NPPB*, *NPR2*, *NXN*, *OBFC2B*, *OGFR*, *ONECUT2*, *OTOP1*, *OXT*, *PACSIN1*, *PAOX*, *PARP3*, *PAX1*, *PCDH8*, *PCDHAC2*, *PDE4C*, *PF4V1*, *PKDREJ*, *PM20D1*, *POMC*, *POU3F1*, *PPAPDC3*, *PRIC285*, *PRLH*, *PSMD11*, *PTGIS*, *RAB36*, *RAP1GAP*, *RASGRF1*, *RASIP1*, *RBPJL*, *RLN1*, *RPL36*, *RPL36AL*, *RPUSD3*, *SCG5*, *SCMH1*, *SCUBE3*, *SEMA3B*, *SGPP2*, *SHROOM1*, *SKAP1*, *SLC12A8*, *SLC5A8*, *SNN*, *SORBS3*, *SPG7*, *SPINT1*, *SRD5A2*, *SRRT*, *SSTR4*, *STMN1*, *TBC1D1*, *TCEA2*, *TCF15*, *TFAP2E*, *TGFBI*, *TIAM1*, *TMEM125*, *TMEM151A*, *TMEM184A*, *TMEM189*, *TMOD3*, *TNNT1*, *TP53INP1*, *TRPC4*, *TRPM3*, *UNC80*, *VAMP5*, *VHL*, *VSTM1*, *WBSCR27*, *WDR52*, *WT1*, *ZFP41*, *ZNF205*, and *ZNF710*.

[00118] In one embodiment, provided herein are response biomarkers for assessing clinical benefit or predicting long-term clinical response, after the initiation of a 5-azacytidine treatment (*e.g.*, assessing clinical benefit or potential long-term clinical response in a patient after or during treatment with 5-azacytidine). In one embodiment, provided herein are methods of using a response biomarker provided herein (*e.g.*, changes in DNA methylation of specific genes). In one embodiment, the clinical benefit includes, but is not limited to, prolonged survival, delayed progression to AML, and/or other beneficial clinical responses. For example, DNA methylation in post-treatment samples may be compared to baseline samples (*e.g.*, after a treatment cycle of about 1, about 2, about 3, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 11, about 12, or greater than about 12 months; or after a treatment cycle of about 1, about 2, about 3, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 12, about 14, about 16, about 18, about 20, about 22, about 24, about 26, about 28, about 30, about 32, about 34, about 36, about 38, about 40, about 42,

about 44, about 46, about 48, about 50, about 52, about 54, about 56, or greater than about 56 weeks). In one embodiment, the methylation levels of particular genes, shown to be methylated in a high proportion at baseline, are monitored periodically after the initiation of a 5-azacytidine treatment.

[00119] In one embodiment, provided herein are biomarkers that could be used to predict which cancer patients will have the most, or least, clinical benefit from a particular cancer therapy. In one embodiment, the methods or biomarkers provided herein may be applied to cancers, such as, *e.g.*, hematological cancers, blood-borne cancers, and/or solid cancers, or a type of cancer described herein elsewhere. *See, e.g.,* International Patent Application No. PCT/US2010/000361, filed February 9, 2010, published as WO2010/093435, incorporated herein by reference in its entirety. In one embodiment, provided herein are biomarkers that could be used to predict which MDS patients will have the most, or least, clinical benefit from a particular MDS therapy, including a therapy for higher-risk MDS. In one embodiment, the methods or biomarkers provided herein may be applied to MDS, *e.g.*, higher-risk MDS. In one embodiment, the biomarkers provided herein are gene methylation biomarkers (*e.g.*, DNA methylation of a particular gene or a particular locus). In other embodiments, methylation level or methylation pattern of other type(s) of nucleic acid(s), *e.g.*, RNA methylation, may be used as a biomarker in a method described herein.

[00120] In one embodiment, provided herein is a method of using DNA methylation (*e.g.*, multiple loci or various single locus) as a pre-treatment biomarker to distinguish patients having potentially greater or lesser response(s) to a particular therapy. In one embodiment, provided herein is a method of using DNA methylation (*e.g.*, multiple loci or various single locus) as a biomarker to predict or monitor the efficacy of a particular cancer treatment. In one embodiment, provided herein is a method of using DNA methylation (*e.g.*, multiple loci or various single locus) as a biomarker to predict or monitor the efficacy of a particular MDS treatment. In one embodiment, provided herein is a method of using DNA methylation (*e.g.*, multiple loci or various single locus) as a biomarker to predict or monitor patient response to a particular cancer treatment. In one embodiment, provided herein is a method of using DNA methylation (*e.g.*, multiple loci or various single locus) as a biomarker to predict or monitor patient response to a particular MDS treatment.

[00121] For example, bio-samples can be obtained from patients having a certain disease (*e.g.*, bone marrow samples obtained from patients having higher-risk MDS; however, it is understood that other bio-samples, *e.g.*, blood or tissue samples, may be used in a method provided herein). In one embodiment, DNA methylation levels of one or more gene(s) or

locus/loci are measured for a particular patient and compared with reference values. In one embodiment, DNA methylation levels of a group of genes are measured for a particular patient and compared with one or more reference methylation pattern(s). In one embodiment, patients are grouped or selected based on DNA methylation level(s) of one or more gene(s) (*e.g.*, a gene or a group of genes described herein elsewhere). In one embodiment, selected patients are further treated with a particular therapy to derive maximal response or clinical benefit, *e.g.*, prolonged overall survival and/or time to AML transformation in MDS.

[00122] In specific embodiments, provided herein is a method of using DNA methylation of one or more gene(s) (*e.g.*, multiple loci or various single locus) as a pre-treatment biomarker to distinguish MDS patients having potentially greater or lesser response to or overall survival benefit from 5-azacytidine therapy.

[00123] In one embodiment, bio-samples (*e.g.*, unpurified bone marrow aspirates) are obtained from patients pre-treatment (*e.g.*, from higher-risk MDS patients before receiving certain treatment). In one embodiment, DNA methylation level of one or more gene(s) provided herein is measured. In one embodiment, DNA methylation of particular gene(s) of a patient is compared with reference value(s). In one embodiment, DNA methylation of a particular group of genes is measured. In one embodiment, DNA methylation pattern of a particular group of genes of a patient is compared with reference pattern(s). In one embodiment, a particular methylation level or a particular methylation pattern is used to distinguish patients having potentially greater or lesser response to or overall survival benefit from a particular therapy (*e.g.*, 5-azacytidine therapy). In one embodiment, a particular group of MDS patients selected based on a method provided herein is treated with 5-azacytidine.

[00124] In one embodiment, provided herein is a method of using gene methylation (*e.g.*, DNA methylation of specific gene(s)) as a predictive biomarker of clinical response or overall survival in MDS patients (*e.g.*, higher-risk MDS patients) treated with a cytidine analog provided herein. In one embodiment, provided herein is a method of using gene methylation as a predictive biomarker of clinical response or overall survival or time to AML transformation in higher-risk MDS patients treated with 5-azacytidine.

[00125] In one embodiment, provided herein is a method of using increased DNA methylation of one or more gene(s), including, but not limited to, *CDKN2B (p15)*, *SOCS1*, *CDH1 (E-cadherin)*, *TP73*, and *CTNNA1 (α-catenin)*, as a predictive biomarker for worse overall survival in patients. In one embodiment, provided herein is a method of using lower levels of methylation of one or more gene(s), including, but not limited to, *CDKN2B (p15)*, *SOCS1*, *CDH1 (E-cadherin)*, *TP73*, and *CTNNA1 (α-catenin)*, as a predictive biomarker for

better overall survival benefit from 5-azacytidine therapy. In one embodiment, the biomarkers of the methods provided herein relate to DNA methylation of one or more gene(s), including, but not limited to, *CDKN2B (p15)*, *SOCS1*, *CDH1 (E-cadherin)*, *TP73*, and *CTNNA1 (α-catenin)*, in pre-treatment baseline bone marrows of MDS. In one embodiment, provided herein is a method of selecting patients having better predicted overall survival benefit using a biomarker provided herein. In one embodiment, the method further comprises administering a therapeutically effective amount of 5-azacytidine to the selected patients.

[00126] In one embodiment, provided herein is a method of using DNA methylation of one or more gene(s), including, but not limited to, *ABHD14A*, *ABO*, *ADAMTS18*, *ADRA2B*, *ADRB3*, *AIRE*, *AKAP12*, *ALOX15B*, *ALS2CR11*, *AMT*, *ANKRD33*, *APC2*, *AVP*, *BHMT*, *C18orf22*, *C19orf30*, *C1orf172*, *C1orf87*, *C3orf15*, *C1QTNF6*, *C22orf27*, *C7orf16*, *C7orf41*, *CBX7*, *CCDC19*, *CCDC81*, *CD164L2*, *CDH1*, *CDKN2B*, *CHAD*, *CHRNG*, *CIDEB*, *CKMT1B*, *CKMT2*, *CLCN6*, *CLDN6*, *CLDN9*, *CNTN4*, *CPT1B*, *CRHBP*, *CXCL5*, *CYP26C1*, *CYP2E1*, *DES*, *DPYS*, *DYDC1*, *EGFL7*, *ELMO3*, *ENTPD2*, *ENTPD3*, *ESR1*, *EYA4*, *F2RL2*, *FAM57B*, *FBLN1*, *FBXO2*, *FKBP1B*, *FLJ44881*, *FLVCR2*, *FREQ*, *FZD9*, *GAB1*, *GAS2L2*, *GATA4*, *GBGT1*, *GDF5*, *GHSR*, *GNAS*, *GNMT*, *GNPNAT1*, *GP1BA*, *GPR25*, *GRM6*, *GSTM5*, *HCN4*, *HIST1H1A*, *HOXD4*, *HSPA2*, *HTATIP2*, *HTR7*, *HYDIN*, *IGDCC3*, *ILDR1*, *IRF6*, *KAZALD1*, *KCNA6*, *KCNK3*, *KCNQ1*, *KIAA0427*, *KIR3DX1*, *KRT25*, *KRT7*, *KRT72*, *LAD1*, *LAMA4*, *LAMC2*, *LGTN*, *LRRC17*, *LTF*, *MBD3L1*, *MEGF10*, *MICAL1*, *MRPL28*, *MTMR9*, *MTNR1B*, *NALCN*, *NCAN*, *NCOR2*, *NDRG2*, *NDUFAF3*, *NEUROG1*, *NGB*, *NPFFR2*, *NPM2*, *NPPB*, *NPR2*, *NXN*, *OBFC2B*, *OGFR*, *ONECUT2*, *OTOP1*, *OXT*, *PACSIN1*, *PAOX*, *PARP3*, *PAX1*, *PCDH8*, *PCDHAC2*, *PDE4C*, *PF4V1*, *PKDREJ*, *PM20D1*, *POMC*, *POU3F1*, *PPAPDC3*, *PRIC285*, *PRLH*, *PSMD11*, *PTGIS*, *RAB36*, *RAP1GAP*, *RASGRF1*, *RASIP1*, *RBPJL*, *RLN1*, *RPL36*, *RPL36AL*, *RPUSD3*, *SCG5*, *SCMH1*, *SCUBE3*, *SEMA3B*, *SGPP2*, *SHROOM1*, *SKAP1*, *SLC12A8*, *SLC5A8*, *SNN*, *SORBS3*, *SPG7*, *SPINT1*, *SRD5A2*, *SRRT*, *SSTR4*, *STMN1*, *TBC1D1*, *TCEA2*, *TCF15*, *TFAP2E*, *TGFBI*, *TIAM1*, *TMEM125*, *TMEM151A*, *TMEM184A*, *TMEM189*, *TMOD3*, *TNNT1*, *TP53INP1*, *TRPC4*, *TRPM3*, *UNC80*, *VAMP5*, *VHL*, *VSTM1*, *WBSCR27*, *WDR52*, *WT1*, *ZFP41*, *ZNF205*, and *ZNF710*, as a predictive biomarker of better or worse overall survival in patients (*e.g.*, after treatment with 5-azacytidine). In one embodiment, provided herein is a method of using DNA methylation of one or more genes(s), including, but not limited to, *WT1*, *CDKN2B*, and/or *CDH1*, as a predictive biomarker of better or worse overall survival in patients (*e.g.*, after treatment with 5-azacytidine). In one embodiment, provided herein is a method of using

DNA methylation of one or more genes(s), including, but not limited to, *WT1*, as a predictive biomarker of better or worse overall survival in patients (*e.g.*, after treatment with 5-azacytidine).

[00127] In one embodiment, provided herein is a method of using DNA methylation of one or more gene(s), including, but not limited to, *ABHD14A*, *ABO*, *ADAMTS18*, *ADRA2B*, *ADRB3*, *AIRE*, *AKAP12*, *ALOX15B*, *ALS2CR11*, *AMT*, *ANKRD33*, *APC2*, *AVP*, *BHMT*, *C18orf22*, *C19orf30*, *C1orf172*, *C1orf87*, *C3orf15*, *C1QTNF6*, *C22orf27*, *C7orf16*, *C7orf41*, *CBX7*, *CCDC19*, *CCDC81*, *CD164L2*, *CDH1*, *CDKN2B*, *CHAD*, *CHRNG*, *CIDEB*, *CKMT1B*, *CKMT2*, *CLCN6*, *CLDN6*, *CLDN9*, *CNTN4*, *CPT1B*, *CRHBP*, *CXCL5*, *CYP26C1*, *CYP2E1*, *DES*, *DPYS*, *DYDC1*, *EGFL7*, *ELMO3*, *ENTPD2*, *ENTPD3*, *ESR1*, *EYA4*, *F2RL2*, *FAM57B*, *FBLN1*, *FBXO2*, *FKBP1B*, *FLJ44881*, *FLVCR2*, *FREQ*, *FZD9*, *GAB1*, *GAS2L2*, *GATA4*, *GBGT1*, *GDF5*, *GHSR*, *GNAS*, *GNMT*, *GNPNAT1*, *GP1BA*, *GPR25*, *GRM6*, *GSTM5*, *HCN4*, *HIST1H1A*, *HOXD4*, *HSPA2*, *HTATIP2*, *HTR7*, *HYDIN*, *IGDCC3*, *ILDR1*, *IRF6*, *KAZALD1*, *KCNA6*, *KCNK3*, *KCNQ1*, *KIAA0427*, *KIR3DX1*, *KRT25*, *KRT7*, *KRT72*, *LAD1*, *LAMA4*, *LAMC2*, *LGTN*, *LRRC17*, *LTF*, *MBD3L1*, *MEGF10*, *MICAL1*, *MRPL28*, *MTMR9*, *MTNR1B*, *NALCN*, *NCAN*, *NCOR2*, *NDRG2*, *NDUFAF3*, *NEUROG1*, *NGB*, *NPFFR2*, *NPM2*, *NPPB*, *NPR2*, *NXN*, *OBFC2B*, *OGFR*, *ONECUT2*, *OTOP1*, *OXT*, *PACSIN1*, *PAOX*, *PARP3*, *PAX1*, *PCDH8*, *PCDHAC2*, *PDE4C*, *PF4V1*, *PKDREJ*, *PM20D1*, *POMC*, *POU3F1*, *PPAPDC3*, *PRIC285*, *PRLH*, *PSMD11*, *PTGIS*, *RAB36*, *RAP1GAP*, *RASGRF1*, *RASIP1*, *RBPJL*, *RLN1*, *RPL36*, *RPL36AL*, *RPUSD3*, *SCG5*, *SCMH1*, *SCUBE3*, *SEMA3B*, *SGPP2*, *SHROOM1*, *SKAP1*, *SLC12A8*, *SLC5A8*, *SNN*, *SORBS3*, *SPG7*, *SPINT1*, *SRD5A2*, *SRRT*, *SSTR4*, *STMN1*, *TBC1D1*, *TCEA2*, *TCF15*, *TFAP2E*, *TGFBI*, *TIAM1*, *TMEM125*, *TMEM151A*, *TMEM184A*, *TMEM189*, *TMOD3*, *TNNT1*, *TP53INP1*, *TRPC4*, *TRPM3*, *UNC80*, *VAMP5*, *VHL*, *VSTM1*, *WBSCR27*, *WDR52*, *WT1*, *ZFP41*, *ZNF205*, and *ZNF710*, as a predictive biomarker of better or worse overall survival benefit from a drug therapy (*e.g.,* administering a cytidine analog to a subject in need thereof, in one embodiment, the cytidine analog is 5-azacytidine). In one embodiment, provided herein is a method of using DNA methylation of one or more gene(s), including, but not limited to, *WT1*, *CDKN2B*, and/or *CDH1*, as a predictive biomarker of better or worse overall survival benefit from a drug therapy (*e.g.,* administering a cytidine analog to a subject in need thereof, in one embodiment, the cytidine analog is 5-azacytidine). In one embodiment, provided herein is a method of using DNA methylation of one or more gene(s), including, but not limited to, *WT1*, as a predictive biomarker of better or worse overall survival benefit from a

drug therapy (*e.g.,* administering a cytidine analog to a subject in need thereof, in one embodiment, the cytidine analog is 5-azacytidine).

[00128] In one embodiment, provided herein is a method of treating MDS, comprising determining DNA methylation of one or more gene(s), including, but not limited to, *ABHD14A*, *ABO*, *ADAMTS18*, *ADRA2B*, *ADRB3*, *AIRE*, *AKAP12*, *ALOX15B*, *ALS2CR11*, *AMT*, *ANKRD33*, *APC2*, *AVP*, *BHMT*, *C18orf22*, *C19orf30*, *C1orf172*, *C1orf87*, *C3orf15*, *C1QTNF6*, *C22orf27*, *C7orf16*, *C7orf41*, *CBX7*, *CCDC19*, *CCDC81*, *CD164L2*, *CDH1*, *CDKN2B*, *CHAD*, *CHRNG*, *CIDEB*, *CKMT1B*, *CKMT2*, *CLCN6*, *CLDN6*, *CLDN9*, *CNTN4*, *CPT1B*, *CRHBP*, *CXCL5*, *CYP26C1*, *CYP2E1*, *DES*, *DPYS*, *DYDC1*, *EGFL7*, *ELMO3*, *ENTPD2*, *ENTPD3*, *ESR1*, *EYA4*, *F2RL2*, *FAM57B*, *FBLN1*, *FBXO2*, *FKBP1B*, *FLJ44881*, *FLVCR2*, *FREQ*, *FZD9*, *GAB1*, *GAS2L2*, *GATA4*, *GBGT1*, *GDF5*, *GHSR*, *GNAS*, *GNMT*, *GNPNAT1*, *GP1BA*, *GPR25*, *GRM6*, *GSTM5*, *HCN4*, *HIST1H1A*, *HOXD4*, *HSPA2*, *HTATIP2*, *HTR7*, *HYDIN*, *IGDCC3*, *ILDR1*, *IRF6*, *KAZALD1*, *KCNA6*, *KCNK3*, *KCNQ1*, *KIAA0427*, *KIR3DX1*, *KRT25*, *KRT7*, *KRT72*, *LAD1*, *LAMA4*, *LAMC2*, *LGTN*, *LRRC17*, *LTF*, *MBD3L1*, *MEGF10*, *MICAL1*, *MRPL28*, *MTMR9*, *MTNR1B*, *NALCN*, *NCAN*, *NCOR2*, *NDRG2*, *NDUFAF3*, *NEUROG1*, *NGB*, *NPFFR2*, *NPM2*, *NPPB*, *NPR2*, *NXN*, *OBFC2B*, *OGFR*, *ONECUT2*, *OTOP1*, *OXT*, *PACSIN1*, *PAOX*, *PARP3*, *PAX1*, *PCDH8*, *PCDHAC2*, *PDE4C*, *PF4V1*, *PKDREJ*, *PM20D1*, *POMC*, *POU3F1*, *PPAPDC3*, *PRIC285*, *PRLH*, *PSMD11*, *PTGIS*, *RAB36*, *RAP1GAP*, *RASGRF1*, *RASIP1*, *RBPJL*, *RLN1*, *RPL36*, *RPL36AL*, *RPUSD3*, *SCG5*, *SCMH1*, *SCUBE3*, *SEMA3B*, *SGPP2*, *SHROOM1*, *SKAP1*, *SLC12A8*, *SLC5A8*, *SNN*, *SORBS3*, *SPG7*, *SPINT1*, *SRD5A2*, *SRRT*, *SSTR4*, *STMN1*, *TBC1D1*, *TCEA2*, *TCF15*, *TFAP2E*, *TGFBI*, *TIAM1*, *TMEM125*, *TMEM151A*, *TMEM184A*, *TMEM189*, *TMOD3*, *TNNT1*, *TP53INP1*, *TRPC4*, *TRPM3*, *UNC80*, *VAMP5*, *VHL*, *VSTM1*, *WBSCR27*, *WDR52*, *WT1*, *ZFP41*, *ZNF205*, and *ZNF710*, in pre-treatment MDS patients (*e.g.*, gene methylation in baseline bone marrows); and selecting patients having better predicted overall survival benefit. In one embodiment, provided herein is a method of treating MDS, comprising determining DNA methylation of one or more gene(s), including, but not limited to, *WT1*, *CDKN2B*, and/or *CDH1*, in pre-treatment MDS patients (*e.g.*, gene methylation in baseline bone marrows); and selecting patients having better predicted overall survival benefit. In one embodiment, provided herein is a method of treating MDS, comprising determining DNA methylation of one or more gene(s), including, but not limited to, *WT1*, in pre-treatment MDS patients (*e.g.*, gene methylation in baseline bone marrows); and selecting patients having better predicted overall survival benefit. In one embodiment, the method further comprises administering a therapeutically effective amount of a cytidine analog (*e.g.*,

5-azacytidine) to the selected patients. In one embodiment, DNA methylation of one or more gene(s) is measured in patients after receiving drug treatment (e.g., 5-azacytidine treatment). In one embodiment, provided herein is a method of using DNA methylation of one or more genes(s) in treated patient as responsive biomarker to assess clinical response and/or predict long term clinical benefit (e.g., overall survival). In one embodiment, DNA methylation is used as biomarker in a method provided herein. In other embodiments, RNA methylation is used as biomarker in a method provided herein.

[00129] In one embodiment, the biomarkers provided herein comprise methylation of one or more gene(s), such as, any 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23,24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 genes, selected from the group consisting of *ABHD14A*, *ABO*, *ADAMTS18*, *ADRA2B*, *ADRB3*, *AIRE*, *AKAP12*, *ALOX15B*, *ALS2CR11*, *AMT*, *ANKRD33*, *APC2*, *AVP*, *BHMT*, *C18orf22*, *C19orf30*, *C1orf172*, *C1orf87*, *C3orf15*, *C1QTNF6*, *C22orf27*, *C7orf16*, *C7orf41*, *CBX7*, *CCDC19*, *CCDC81*, *CD164L2*, *CDH1*, *CDKN2B*, *CHAD*, *CHRNG*, *CIDEB*, *CKMT1B*, *CKMT2*, *CLCN6*, *CLDN6*, *CLDN9*, *CNTN4*, *CPT1B*, *CRHBP*, *CXCL5*, *CYP26C1*, *CYP2E1*, *DES*, *DPYS*, *DYDC1*, *EGFL7*, *ELMO3*, *ENTPD2*, *ENTPD3*, *ESR1*, *EYA4*, *F2RL2*, *FAM57B*, *FBLN1*, *FBXO2*, *FKBP1B*, *FLJ44881*, *FLVCR2*, *FREQ*, *FZD9*, *GAB1*, *GAS2L2*, *GATA4*, *GBGT1*, *GDF5*, *GHSR*, *GNAS*, *GNMT*, *GNPNAT1*, *GP1BA*, *GPR25*, *GRM6*, *GSTM5*, *HCN4*, *HIST1H1A*, *HOXD4*, *HSPA2*, *HTATIP2*, *HTR7*, *HYDIN*, *IGDCC3*, *ILDR1*, *IRF6*, *KAZALD1*, *KCNA6*, *KCNK3*, *KCNQ1*, *KIAA0427*, *KIR3DX1*, *KRT25*, *KRT7*, *KRT72*, *LAD1*, *LAMA4*, *LAMC2*, *LGTN*, *LRRC17*, *LTF*, *MBD3L1*, *MEGF10*, *MICAL1*, *MRPL28*, *MTMR9*, *MTNR1B*, *NALCN*, *NCAN*, *NCOR2*, *NDRG2*, *NDUFAF3*, *NEUROG1*, *NGB*, *NPFFR2*, *NPM2*, *NPPB*, *NPR2*, *NXN*, *OBFC2B*, *OGFR*, *ONECUT2*, *OTOP1*, *OXT*, *PACSIN1*, *PAOX*, *PARP3*, *PAX1*, *PCDH8*, *PCDHAC2*, *PDE4C*, *PF4V1*, *PKDREJ*, *PM20D1*, *POMC*, *POU3F1*, *PPAPDC3*, *PRIC285*, *PRLH*, *PSMD11*, *PTGIS*, *RAB36*, *RAP1GAP*, *RASGRF1*, *RASIP1*, *RBPJL*, *RLN1*, *RPL36*, *RPL36AL*, *RPUSD3*, *SCG5*, *SCMH1*, *SCUBE3*, *SEMA3B*, *SGPP2*, *SHROOM1*, *SKAP1*, *SLC12A8*, *SLC5A8*, *SNN*, *SORBS3*, *SPG7*, *SPINT1*, *SRD5A2*, *SRRT*, *SSTR4*, *STMN1*, *TBC1D1*, *TCEA2*, *TCF15*, *TFAP2E*, *TGFBI*, *TIAM1*, *TMEM125*, *TMEM151A*, *TMEM184A*, *TMEM189*, *TMOD3*, *TNNT1*, *TP53INP1*, *TRPC4*, *TRPM3*, *UNC80*, *VAMP5*, *VHL*, *VSTM1*, *WBSCR27*, *WDR52*, *WT1*, *ZFP41*, *ZNF205*, and *ZNF710*.

[00130] In one embodiment, the biomarkers provided herein comprise methylation of one or more gene(s), such as, about 5, about 10, about 15, about 20, about 25, about 30, about 35, about 40, about 45, about 50, about 55, about 60, about 65, about 70, about 75, about 80,

about 85, about 90, about 95, about 100, or more than 100 genes, selected from the group consisting of *ABHD14A*, *ABO*, *ADAMTS18*, *ADRA2B*, *ADRB3*, *AIRE*, *AKAP12*, *ALOX15B*, *ALS2CR11*, *AMT*, *ANKRD33*, *APC2*, *AVP*, *BHMT*, *C18orf22*, *C19orf30*, *C1orf172*, *C1orf87*, *C3orf15*, *C1QTNF6*, *C22orf27*, *C7orf16*, *C7orf41*, *CBX7*, *CCDC19*, *CCDC81*, *CD164L2*, *CDH1*, *CDKN2B*, *CHAD*, *CHRNG*, *CIDEB*, *CKMT1B*, *CKMT2*, *CLCN6*, *CLDN6*, *CLDN9*, *CNTN4*, *CPT1B*, *CRHBP*, *CXCL5*, *CYP26C1*, *CYP2E1*, *DES*, *DPYS*, *DYDC1*, *EGFL7*, *ELMO3*, *ENTPD2*, *ENTPD3*, *ESR1*, *EYA4*, *F2RL2*, *FAM57B*, *FBLN1*, *FBXO2*, *FKBP1B*, *FLJ44881*, *FLVCR2*, *FREQ*, *FZD9*, *GAB1*, *GAS2L2*, *GATA4*, *GBGT1*, *GDF5*, *GHSR*, *GNAS*, *GNMT*, *GNPNAT1*, *GP1BA*, *GPR25*, *GRM6*, *GSTM5*, *HCN4*, *HIST1H1A*, *HOXD4*, *HSPA2*, *HTATIP2*, *HTR7*, *HYDIN*, *IGDCC3*, *ILDR1*, *IRF6*, *KAZALD1*, *KCNA6*, *KCNK3*, *KCNQ1*, *KIAA0427*, *KIR3DX1*, *KRT25*, *KRT7*, *KRT72*, *LAD1*, *LAMA4*, *LAMC2*, *LGTN*, *LRRC17*, *LTF*, *MBD3L1*, *MEGF10*, *MICAL1*, *MRPL28*, *MTMR9*, *MTNR1B*, *NALCN*, *NCAN*, *NCOR2*, *NDRG2*, *NDUFAF3*, *NEUROG1*, *NGB*, *NPFFR2*, *NPM2*, *NPPB*, *NPR2*, *NXN*, *OBFC2B*, *OGFR*, *ONECUT2*, *OTOP1*, *OXT*, *PACSIN1*, *PAOX*, *PARP3*, *PAX1*, *PCDH8*, *PCDHAC2*, *PDE4C*, *PF4V1*, *PKDREJ*, *PM20D1*, *POMC*, *POU3F1*, *PPAPDC3*, *PRIC285*, *PRLH*, *PSMD11*, *PTGIS*, *RAB36*, *RAP1GAP*, *RASGRF1*, *RASIP1*, *RBPJL*, *RLN1*, *RPL36*, *RPL36AL*, *RPUSD3*, *SCG5*, *SCMH1*, *SCUBE3*, *SEMA3B*, *SGPP2*, *SHROOM1*, *SKAP1*, *SLC12A8*, *SLC5A8*, *SNN*, *SORBS3*, *SPG7*, *SPINT1*, *SRD5A2*, *SRRT*, *SSTR4*, *STMN1*, *TBC1D1*, *TCEA2*, *TCF15*, *TFAP2E*, *TGFBI*, *TIAM1*, *TMEM125*, *TMEM151A*, *TMEM184A*, *TMEM189*, *TMOD3*, *TNNT1*, *TP53INP1*, *TRPC4*, *TRPM3*, *UNC80*, *VAMP5*, *VHL*, *VSTM1*, *WBSCR27*, *WDR52*, *WT1*, *ZFP41*, *ZNF205*, and *ZNF710*.

5.4.1 Treatment, Prevention, and/or Management

[00131] In one embodiment, provided herein is a method of treating, preventing, or managing cancer, comprising measuring gene methylation of a patient having cancer, and administering a therapeutic agent (*e.g.*, a cytidine analog, e.g. 5-azacytidine) to a patient in need thereof. In one embodiment, the cancer is a blood-borne tumor. In one embodiment, the cancer is a solid tumor.

[00132] In one embodiment, provided herein is a method of treating, preventing, or managing MDS, comprising measuring gene methylation of a patient having MDS, and administering a therapeutic agent (*e.g.*, a cytidine analog, e.g. 5-azacytidine) to a patient in need thereof. In one embodiment, the MDS is higher-risk MDS.

[00133] In one embodiment, patients are screened prior to enrollment in a clinical study or prior to treatment by a physician, for DNA or RNA methylation levels. In one embodiment, patients are monitored during a clinical study or during a treatment course, for DNA or RNA

methylation levels. In one embodiment, DNA or RNA methylation levels of particular gene(s) (*e.g.*, at particular loci/locus) are indicative of a potential response to a treatment (*e.g.*, treatment comprising a cytidine analog, *e.g.*, 5-azacytidine).

[00134] Embodiments further provided herein are methods of treatments with a pharmaceutical composition comprising a cytidine analog, particularly, 5-azacytidine, providing particular benefit to the population of patients stratified into the higher risk groups of myelodysplastic syndromes (MDS) by conventional scoring systems, as measured by improved survival of this population upon treatment with a cytidine analog, *e.g.*, 5-azacytidine. *See, e.g.*, U.S. Patent Application No. 12/740,636, filed November 3, 2008, incorporated herein by reference in its entirety.

[00135] Accordingly, in one embodiment, provided herein is a method of treating a patient diagnosed with a higher risk MDS, the method comprising treating the patient diagnosed with a higher risk MDS with an effective amount of a composition comprising a cytidine analog. In one embodiment, the method further comprises the step of selecting patients using a predictive biomarker provided herein elsewhere, and treating the selected patients with an effective amount of a composition comprising a cytidine analog (*e.g.*, 5-azacytidine).

[00136] In one embodiment, the cytidine analog includes any moiety which is structurally related to cytidine or deoxycytidine and functionally mimics and/or antagonizes the action of cytidine or deoxycytidine. These analogs may also be called cytidine derivatives herein. In one embodiment, cytidine analog includes 5-aza-2'-deoxycytidine (decitabine), 5-azacytidine, 5-aza-2'-deoxy-2',2'-difluorocytidine, 5-aza-2'-deoxy-2'-fluorocytidine, 2'-deoxy-2',2'-difluorocytidine (also called gemcitabine), or cytosine 1-β-D-arabinofuranoside (also called ara-C), 2(1*H*)-pyrimidine-riboside (also called zebularine), 2'-cyclocytidine, arabinofuanosyl-5-azacytidine, dihydro-5-azacytidine, N^4-octadecyl-cytarabine, and elaidic acid cytarabine. In one embodiment, cytidine analog includes 5-azacytidine and 5-aza-2'-deoxycytidine. The definition of cytidine analog used herein also includes mixtures of cytidine analogs.

[00137] Cytidine analogs may be synthesized by methods known in the art. In one embodiment, methods of synthesis include methods as disclosed in U.S. Serial No. 10/390,526 (U.S. Patent No. 7,038,038); U.S. Serial No. 10/390,578 (U.S. Patent No. 6,887,855); U.S. Serial No. 11/052615 (U.S. Patent No. 7,078,518); U.S. Serial No. 10390530 (U.S. Patent No. 6,943,249); and U.S. Serial No. 10/823,394, all incorporated by reference herein in their entireties.

[00138] In one embodiment, an effective amount of a cytidine analog to be used is a therapeutically effective amount. In one embodiment, the amounts of a cytidine analog to be used in the methods provided herein and in the oral formulations include a therapeutically effective amount, typically, an amount sufficient to cause improvement in at least a subset of patients with respect to symptoms, overall course of disease, or other parameters known in the art. Therapeutic indications are discussed more fully herein below. Precise amounts for therapeutically effective amounts of the cytidine analog in the pharmaceutical compositions will vary depending on the age, weight, disease, and condition of the patient. For example, pharmaceutical compositions may contain sufficient quantities of a cytidine analog to provide a daily dosage of about 10 to 150 mg/m^2 (based on patient body surface area) or about 0.1 to 4 mg/kg (based on patient body weight) as single or divided (2–3) daily doses. In one embodiment, dosage is provided via a seven-day administration of 75 mg/m^2 subcutaneously, once every twenty-eight days, for as long as clinically necessary. In one embodiment, dosage is provided via a seven-day administration of 100 mg/m^2 subcutaneously, once every twenty-eight days, for as long as clinically necessary. In one embodiment, up to 4, up to 5, up to 6, up to 7, up to 8, up to 9 or more 28-day cycles are administered. Other methods for providing an effective amount of a cytidine analog are disclosed in, for example, "Colon-Targeted Oral Formulations of Cytidine Analogs", U.S. Serial No. 11/849,958, and "Oral Formulations of Cytidine Analogs and Methods of Use Thereof", U.S. Serial No. 12/466,213, both of which are incorporated by reference herein in their entireties.

[00139] Hematologic disorders include abnormal growth of blood cells which can lead to dysplastic changes in blood cells and hematologic malignancies such as various leukemias. Examples of hematologic disorders include but are not limited to acute myeloid leukemia, acute promyelocytic leukemia, acute lymphoblastic leukemia, chronic myelogenous leukemia, the myelodysplastic syndromes, and sickle cell anemia.

[00140] Acute myeloid leukemia (AML) is the most common type of acute leukemia that occurs in adults. Several inherited genetic disorders and immunodeficiency states are associated with an increased risk of AML. These include disorders with defects in DNA stability, leading to random chormosomal breakage, such as Bloom's syndrome, Fanconi's anemia, Li-Fraumeni kindreds, ataxia-telangiectasia, and X-linked agammaglobulinemia.

[00141] Acute promyelocytic leukemia (APML) represents a distinct subgroup of AML. This subtype is characterized by promyelocytic blasts containing the 15;17 chromosomal translocation. This translocation leads to the generation of the fusion transcript comprised of the retinoic acid receptor and a sequence PML.

[00142] Acute lymphoblastic leukemia (ALL) is a heterogenerous disease with distinct clinical features displayed by various subtypes. Reoccurring cytogenetic abnormalities have been demonstrated in ALL. The most common cytogenetic abnormality is the 9;22 translocation. The resultant Philadelphia chromosome represents poor prognosis of the patient.

[00143] Chronic myelogenous leukemia (CML) is a clonal myeloproliferative disorder of a pluripotent stem cell. CML is characterized by a specific chromosomal abnormality involving the translocation of chromosomes 9 and 22, creating the Philadelphia chromosome. Ionizing radiation is associated with the development of CML.

[00144] The myelodysplastic syndromes (MDS) are heterogeneous clonal hematopoietic stem cell disorders grouped together, because of the presence of dysplastic changes in one or more of the hematopoietic lineages including dysplastic changes in the myeloid, erythroid, and megakaryocytic series. These changes result in cytopenias in one or more of the three lineages. Patients afflicted with MDS typically develop complications related to anemia, neutropenia (infections), or thrombocytopenia (bleeding). Generally, from about 10% to about 70% of patients with MDS develop acute leukemia. MDS affects approximately 40,000-50,000 people in the U.S. and 75,000-85,000 patients in Europe. The majority of people with higher risk MDS eventually experience bone marrow failure. Up to 50% of MDS patients succumb to complications, such as infection or bleeding, before progressing to acute myeloid leukemia (AML). MDS patients have a median survival of four months to five years depending on risk stratification. Higher risk patients have a median survival of five to 14 months. Altering the natural history of the disease and providing increased survival is one of the most important treatment goals in higher risk MDS.

[00145] In one embodiment, MDS is a condition to be treated with methods provided herein, and includes the following MDS subtypes: refractory anemia, refractory anemia with ringed sideroblasts (if accompanied by neutropenia or thrombocytopenia or requiring transfusions), refractory anemia with excess blasts, refractory anemia with excess blasts in transformation, and chronic myelomonocytic leukemia. In another embodiment, the condition to be treated is higher risk MDS.

[00146] In classifying a patient's disease as "higher risk MDS" (also referred to herein as, *e.g.*, "higher-risk MDS," "high risk MDS" and "high-risk MDS"), methods known in the art can be used by the skilled person in order to classify a patient's disease as "higher risk" MDS. Such methods include, *e.g.*, the FAB system, the WHO system, and IPSS, as discussed herein below (*See, e.g.*, Bennett J.M., A comparative review of classification

systems in myelodysplastic syndromes (MDS), *Semin. Oncol.* 2005 Aug; 32(4 Suppl 5):S3-10; Bennett et al., *Br. J. Haematol.* 1982, 51:189-99; Harris et al., *J. Clin. Oncol.* 1999, 17(12):3835-49; Greenberg et al., *Blood* 1997, 89(6), 2079-98). Other methods for such assessment may lie within the knowledge or expertise of the skilled person, and methods provided herein include such a skilled person's assessment.

[00147] The skilled person knows that experience has shown that certain disease factors affect a person's prognosis — his or her chances of long-term survival and risk of developing AML. Researchers use these factors to classify MDS into types. In one embodiment, the system to classify MDS is the FAB system, so-called because it was developed by a team of French, American and British researchers. In the FAB system, there are five types of MDS. The FAB system uses several disease factors to classify MDS. One important factor is the percent of blasts in the bone marrow (Table 1). A higher percent of blasts is linked to a higher likelihood of developing AML and a poorer prognosis. The two more common types of MDS are refractory anemia (RA) and refractory anemia with ringed sideroblasts (RARS). These are also the less severe forms of MDS. They have a lower risk of turning into AML. Some patients with these forms of MDS may live with few symptoms and need little treatment for many years.

[00148] The other types of MDS tend to be more severe and more difficult to treat successfully. The refractory anemia with excess blasts (RAEB) and refractory anemia with excess blasts in transformation (RAEB-t) forms of MDS also have a high risk of turning into AML.

Table 1. MDS Types in the FAB System

Type of MDS	Percent of blasts in marrow (less than 5% is normal)
Refractory anemia (RA)	Less than 5% (normal amount)
Refractory anemia with ringed sideroblasts (RARS)	Less than 5% (normal amount), plus more than 15% of abnormal red blood cells called ringed sideroblasts
Refractory anemia with excess blasts (RAEB)	5% to 20%
Refractory anemia with excess blasts in transformation (RAEB-T)	21% to 30%
Chronic myelomonocytic leukemia (CMML)	5% to 20%, plus a large number of a type of white blood cell called monocytes

[00149] In another embodiment, a system for defining types of MDS is the newer World Health Organization (WHO) system which divides MDS into eight types. (*See, e.g.*, Muller-Berndorff, *et al.*, *Ann. Hematol.* 2006 Aug; 85(8):502-13.) In certain embodiments, a skilled person may use either the FAB or WHO system to determine the type of MDS .

[00150] In another embodiment, individual prognosis is determined using the international prognostic scoring system (IPSS). The IPSS risk score describes the risk that a person's disease will develop into AML or become life-threatening. A doctor may use the IPSS risk score along with the MDS type to plan treatment. The IPSS risk score is based on three factors that have been shown to affect a patient's prognosis:

(1) The percent of cells in the bone marrow that are blasts.

(2) Whether one, two or all three types of blood cells are low (also called cytopenias). The three types are red blood cells, white blood cells, and platelets.

(3) Changes in the chromosomes of bone marrow blood cells. This may be called cytogenetics (the study of chromosome abnormalities). It may also be called the karyotype (a picture of the chromosomes that shows whether they are abnormal).

[00151] A person may have an IPSS risk score of low, intermediate-1, intermediate-2 or high risk. Doctors can use the risk score to plan treatment. Someone with low-risk disease may be likely to survive for years with few symptoms. That person may need less intense treatment. Someone with intermediate-1, intermediate-2 or high-risk disease may be likely to survive only if he or she receives aggressive treatment, such as a transplant.

[00152] In one embodiment, a higher risk patient is treated by the methods provided herein. In one embodiment, a patient defined as a higher risk MDS patient includes those whose disease is assessed as any one or more of the following: RAEB, RAEB-T, or CMML (10-29% marrow blasts) under FAB or with an IPSS of Intermediate-2 or High.

[00153] In one embodiment, dosing schedules for the compositions and methods provided herein, for example, can be adjusted to account for the patient's characteristics and disease status. Appropriate dose will depend on the disease state being treated. In some cases, dosing schedules include daily doses, and in others, selected days of a week, month or other time interval. In one embodiment, the drug will not be given more than once per day. In one embodiment, dosing schedules for administration of pharmaceutical compositions include the daily administration to a patient in need thereof. Dosing schedules may mimic those that are used for non-oral formulations of a cytidine analog, adjusted to maintain, for example, substantially equivalent therapeutic concentration in the patient's body.

[00154] In certain embodiments, appropriate biomarkers may be used to evaluate the drug's effects on the disease state and provide guidance to the dosing schedule. For example, particular embodiments herein provide a method of determining whether a patient diagnosed with MDS has an increased probability of obtaining a greater benefit from treatment with a cytidine analog by assessing the patient's nucleic acid methylation status. In particular embodiments, the cytidine analog is 5-azacytidine. In particular embodiments, the nucleic acid is DNA or RNA. In particular embodiments, the greater benefit is an overall survival benefit. In particular embodiments, the methylation status is examined in one or more genes, *e.g.*, genes associated with MDS or AML. In particular embodiments, the methylation status is examined in one or more genes, *e.g.*, genes described herein elsewhere. Specific embodiments involve methods for determining whether baseline DNA methylation levels influence overall survival in patients with MDS (*e.g.*, higher risk MDS) treated with 5-azacytidine. Specific embodiments provide methods for determining whether gene methylation levels influence overall survival in patients with MDS (*e.g.*, higher risk MDS). Specific embodiments provide methods for determining whether gene promoter methylation levels influence overall survival in patients with MDS (*e.g.*, higher risk MDS).

[00155] For example, specific embodiments herein provide methods for evaluating the influence of gene methylation on prolonged survival in patients with MDS (*e.g.*, higher risk MDS). In particular embodiments, such evaluation is used to predict overall survival in patients with MDS (*e.g.*, higher risk MDS), *e.g.*, upon treatment with 5-azacytidine. In particular embodiments, such evaluation is used for therapeutic decision-making. In specific embodiments, such therapeutic decision-making includes planning or adjusting a patient's treatment, *e.g.*, the dosing regimen, amount, and/or duration of 5-azacytidine administration.

[00156] Certain embodiments provide methods of identifying individual patients diagnosed with MDS having an increased probability of obtaining an overall survival benefit from 5-azacytidine treatment, using analysis of methylation levels, *e.g.*, in particular genes.

[00157] In some embodiments, lower levels of nucleic acid methylation (*e.g.*, of certain genes) are associated with an increased probability of obtaining improved overall survival following 5-azacytidine treatment.

[00158] In some embodiments, particular patterns of nucleic acid methylation (*e.g.*, particular gene methylation signature) are associated with an increased probability of obtaining improved overall survival following 5-azacytidine treatment.

[00159] In particular embodiments, the increased probability of obtaining improved overall survival following 5-azacytidine treatment is at least a 5% greater probability, at least

a 10% greater probability, at least a 20% greater probability, at least a 30% greater probability, at least a 40% greater probability, at least a 50% greater probability, at least a 60% greater probability, at least a 70% greater probability, at least an 80% greater probability, at least a 90% greater probability, at least at least a 100% greater probability, at least a 125% greater probability, at least a 150% greater probability, at least a 175% greater probability, at least a 200% greater probability, at least a 250% greater probability, at least a 300% greater probability, at least a 400% greater probability, or at least a 500% greater probability of obtaining improved overall survival following 5-azacytidine treatment. In particular embodiments, the greater probability of obtaining improved overall survival following 5-azacytidine treatment is a greater probability as compared to the average probability of a particular comparison population of patients diagnosed with MDS. In specific embodiments, the comparison population is a group of patients classified with a particular myelodysplastic subtype, as described herein. In one embodiment, the comparison population consists of patients having higher risk MDS. In particular embodiments, the comparison population consists of a particular IPSS cytogenetic subgroup.

[00160] In particular embodiments, nucleic acid (*e.g.*, DNA or RNA) hypermethylation status may be determined by any method known in the art. In certain embodiments, DNA hypermethylation status may be determined using the bone marrow aspirates of patients diagnosed with MDS, *e.g.*, by using quantitative real-time methylation specific PCR ("qMSP"). In certain embodiments, the methylation analysis may involve bisulfite conversion of genomic DNA. For example, in certain embodiments, bisulfite treatment of DNA is used to convert non-methylated CpG sites to UpG, leaving methylated CpG sites intact. *See, e.g.*, Frommer, M., *et al.*, *Proc. Nat'l Acad. Sci. USA* 1992, 89:1827-31. Commercially available kits may be used for such bisulfite treatment. In certain embodiments, to facilitate methylation PCR, primers are designed as known in the art, *e.g.*, outer primers which amplify DNA regardless of methylation status, and nested primers which bind to methylated or non-methylated sequences within the region amplified by the first PCR. *See, e.g.*, Li *et al.*, *Bioinformatics* 2002, 18:1427-31. In certain embodiments, probes are designed, *e.g.*, probes which bind to the bisulfite-treated DNA regardless of methylation status. In certain embodiments, CpG methylation is detected, *e.g.*, following PCR amplification of bisulfite-treated DNA using outer primers. In certain embodiments, amplified product from the initial PCR reaction serves as a template for the nested PCR reaction using methylation-specific primers or non-methylation-specific primers. In certain embodiments, a standard curve is established to determine the percentage of methylated

molecules in a particular sample. Methods for detecting nucleic acid methylation (*e.g.*, RNA or DNA methylation) are known in art. *See, e.g.*, Laird, P.W., *Nature Rev. Cancer* 2003, 3:253-66; Belinsky, S.A., *Nature Rev. Cancer* 2004, 4:1-11.

[00161] In certain embodiments, statistical analyses are performed to assess the influence of particular methylation levels with the potential benefit of treatment with a particular cytidine analog. In certain embodiments, the influence of methylation on overall survival is assessed, *e.g.*, using Cox proportional hazards models and Kaplan-Meier (KM) methodology.

[00162] In one embodiment, a gene associated with MDS and/or AML may be examined for its methylation status in a patient. In one embodiment, particular genes include, but are not limited to, *CDKN2B (p15)*, *SOCS1*, *CDH1 (E-cadherin)*, *TP73*, and *CTNNA1 (alpha-catenin)*. In some embodiments, some of the genes associated with MDS and/or AML, which may be suitable for use in the methods disclosed here, are known in the art.

[00163] In one embodiment, particular genes that may be examined for its methylation status in a patient, include, but are not limited to, *ABHD14A*, *ABO*, *ADAMTS18*, *ADRA2B*, *ADRB3*, *AIRE*, *AKAP12*, *ALOX15B*, *ALS2CR11*, *AMT*, *ANKRD33*, *APC2*, *AVP*, *BHMT*, *C18orf22*, *C19orf30*, *C1orf172*, *C1orf87*, *C3orf15*, *C1QTNF6*, *C22orf27*, *C7orf16*, *C7orf41*, *CBX7*, *CCDC19*, *CCDC81*, *CD164L2*, *CDH1*, *CDKN2B*, *CHAD*, *CHRNG*, *CIDEB*, *CKMT1B*, *CKMT2*, *CLCN6*, *CLDN6*, *CLDN9*, *CNTN4*, *CPT1B*, *CRHBP*, *CXCL5*, *CYP26C1*, *CYP2E1*, *DES*, *DPYS*, *DYDC1*, *EGFL7*, *ELMO3*, *ENTPD2*, *ENTPD3*, *ESR1*, *EYA4*, *F2RL2*, *FAM57B*, *FBLN1*, *FBXO2*, *FKBP1B*, *FLJ44881*, *FLVCR2*, *FREQ*, *FZD9*, *GAB1*, *GAS2L2*, *GATA4*, *GBGT1*, *GDF5*, *GHSR*, *GNAS*, *GNMT*, *GNPNAT1*, *GP1BA*, *GPR25*, *GRM6*, *GSTM5*, *HCN4*, *HIST1H1A*, *HOXD4*, *HSPA2*, *HTATIP2*, *HTR7*, *HYDIN*, *IGDCC3*, *ILDR1*, *IRF6*, *KAZALD1*, *KCNA6*, *KCNK3*, *KCNQ1*, *KIAA0427*, *KIR3DX1*, *KRT25*, *KRT7*, *KRT72*, *LAD1*, *LAMA4*, *LAMC2*, *LGTN*, *LRRC17*, *LTF*, *MBD3L1*, *MEGF10*, *MICAL1*, *MRPL28*, *MTMR9*, *MTNR1B*, *NALCN*, *NCAN*, *NCOR2*, *NDRG2*, *NDUFAF3*, *NEUROG1*, *NGB*, *NPFFR2*, *NPM2*, *NPPB*, *NPR2*, *NXN*, *OBFC2B*, *OGFR*, *ONECUT2*, *OTOP1*, *OXT*, *PACSIN1*, *PAOX*, *PARP3*, *PAX1*, *PCDH8*, *PCDHAC2*, *PDE4C*, *PF4V1*, *PKDREJ*, *PM20D1*, *POMC*, *POU3F1*, *PPAPDC3*, *PRIC285*, *PRLH*, *PSMD11*, *PTGIS*, *RAB36*, *RAP1GAP*, *RASGRF1*, *RASIP1*, *RBPJL*, *RLN1*, *RPL36*, *RPL36AL*, *RPUSD3*, *SCG5*, *SCMH1*, *SCUBE3*, *SEMA3B*, *SGPP2*, *SHROOM1*, *SKAP1*, *SLC12A8*, *SLC5A8*, *SNN*, *SORBS3*, *SPG7*, *SPINT1*, *SRD5A2*, *SRRT*, *SSTR4*, *STMN1*, *TBC1D1*, *TCEA2*, *TCF15*, *TFAP2E*, *TGFBI*, *TIAM1*, *TMEM125*, *TMEM151A*, *TMEM184A*, *TMEM189*, *TMOD3*, *TNNT1*, *TP53INP1*, *TRPC4*, *TRPM3*, *UNC80*, *VAMP5*, *VHL*, *VSTM1*, *WBSCR27*, *WDR52*, *WT1*, *ZFP41*, *ZNF205*, and *ZNF710*. In one embodiment, particular genes that may be examined for its methylation

status in a patient, include, but are not limited to, *WT1*, *CDKN2B*, and *CDH1*. In one embodiment, particular gene that may be examined for its methylation status in a patient, includes, but is not limited to, *WT1*. In certain embodiments, particular gene methylation patterns or methylation signatures as disclosed herein elsewhere are suitable for use in the methods disclosed herein.

[00164] In another embodiment, provided herein is a method of selecting a patient diagnosed with MDS for treatment with 5-azacytidine, comprising assessing a patient diagnosed with MDS for having a particular gene methylation profile or pattern, and selecting a patient for treatment with 5-azacytidine where the patient's gene methylation profile is predicted or assessed as having greater clinical response to 5-azacytidine (*e.g.*, prolonged overall survival or prolonged time to AML transformation). In another embodiment, provided herein is a method to improve survival in a patient population with higher risk MDS, the method comprising treating at least one patient diagnosed with a higher risk MDS with an effective amount of a composition comprising a cytidine analog. In one embodiment, the method comprises selecting patients based on their gene methylation profiles, prior to the initiation of treatment.

[00165] Certain embodiments herein provide methods for the treatment of MDS. In certain embodiments, the methods comprise providing for the survival of an MDS patient beyond a specific period of time by administering a specific dose of 5-azacytidine for at least a specific number of cycles of 5-azacytidine treatment. In particular embodiments, the contemplated specific period of time for survival is, *e.g.*, beyond 10 months, beyond 11 months, beyond 12 months, beyond 13 months, beyond 14 months, beyond 15 months, beyond 16 months, beyond 17 months, beyond 18 months, beyond 19 months, or beyond 20 months. In particular embodiments, the contemplated specific number of cycles administered is, *e.g.*, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, at least 22, at least 24, at least 26, at least 28, at least 30, at least 32, at least 34, at least 36, at least 38, at least 40, at least 42, at least 44, at least 46, at least 48, or at least 50 cycles of 5-azacytidine treatment. In particular embodiments, the contemplated treatment is administered, *e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14 days out of a 28-day period. In particular embodiments, the contemplated specific 5-azacytidine dose is, *e.g.*, at least at least 10 mg/day, at least 20 mg/day, at least 30 mg/day, at least 40 mg/day, at least 50 mg/day, at least 55 mg/day, at least 60 mg/day, at least 65 mg/day, at least 70 mg/day, at least 75 mg/day, at least 80 mg/day, at least 85 mg/day, at least 90 mg/day, at least 95 mg/day, or at

least 100 mg/day. In particular embodiments, the dosing is performed, *e.g.*, subcutaneously or intravenously. In particular embodiments, the contemplated specific 5-azacytidine dose is, *e.g.*, at least 50 mg/m^2/day, at least 60 mg/m^2/day, at least 70 mg/m^2/day, at least 75 mg/m^2/day, at least 80 mg/m^2/day, at least 90 mg/m^2/day, or at least 100 mg/m^2/day. One particular embodiment herein provides a method for obtaining the survival of an MDS patient beyond 15 months by administering at least 9 cycles of 5-azacytidine treatment. One particular embodiment herein provides administering the treatment for 7 days out of each 28-day period. One particular embodiment herein provides a dosing regimen of 75 mg/m^2 subcutaneously or intravenously, daily for 7 days. One particular embodiment herein provides a dosing regimen of 100 mg/m^2 subcutaneously or intravenously, daily for 7 days.

[00166] Particular embodiments provide treating a subject having MDS using one or more of the methods provided herein, together with surgery. Particular embodiments provide treating a subject having MDS using one or more of the methods provided herein, together with chemotherapy. Particular embodiments provide treating a subject having MDS using one or more of the methods provided herein, together with immunotherapy. Particular embodiments provide treating a subject having MDS using one or more of the methods provided herein, together with targeted therapy. Particular embodiments provide treating a subject having MDS using one or more of the methods provided herein, together with radiation therapy. Particular embodiments provide treating a subject having MDS using one or more of the methods provided herein, together with two or more of the treatments selected from surgery, chemotherapy, immunotherapy, targeted therapy, and radiation therapy. Particular embodiments provide treating a subject having MDS using one or more of the methods provided herein, together with two or more of the treatments selected from chemotherapy, immunotherapy, radiation, and targeted therapy.

[00167] In certain embodiments, the subject to be treated with one of the methods provided herein has not been treated with an anticancer or anti-MDS therapy prior to the administration of the cytidine analog. In certain embodiments, the subject to be treated with one of the methods provided herein has been treated with one or more anticancer or anti-MDS therapies prior to the administration of the cytidine analog. In certain embodiments, the subject to be treated with one of the methods provided herein has been treated with a cancer therapeutic agent or a MDS therapeutic agent. In certain embodiments, the subject to be treated with one of the methods provided herein has developed drug resistance to anticancer or anti-MDS therapy. In certain embodiments, the subject to be treated with the methods provided herein has a relapsed cancer. In certain embodiments, the subject to be treated with

the methods provided herein has a refractory cancer. In certain embodiments, the subject to be treated with the methods provided herein has a metastatic cancer. In certain embodiments, the subject to be treated with the methods provided herein has a high-risk MDS. In certain embodiments, the subject to be treated with the methods provided herein has a higher-risk MDS.

[00168] In one embodiment, the methods provided herein encompass treating a subject regardless of patient's age, although some diseases or disorders are more common in certain age groups. Further provided herein is a method for treating a subject who has undergone surgery in an attempt to treat the disease or condition at issue. Further provided herein is a method for treating a subject who has not undergone surgery as an attempt to treat the disease or condition at issue. In some embodiments, because the subjects with cancer have heterogeneous clinical manifestations and varying clinical outcomes, the treatment given to a particular subject may vary, depending on his/her prognosis. In some embodiments, the skilled clinician may be able to readily determine without undue experimentation, specific secondary agents, types of surgery, and types of non-drug based standard therapy that can be effectively used to treat an individual subject with cancer or MDS.

[00169] In each embodiment provided herein, the method may further comprise one or more diagnostic steps, to determine, *e.g.*, the type of MDS or cancer, the presence of particular gene methylation profile, and/or the staging of the disease in a subject.

[00170] In each embodiment provided herein, the method may further comprise a disease evaluation step after the cytidine analog has been administered to the subject, to determine, *e.g.*, changes in one or more molecular markers as described herein elsewhere, and/or other benchmarks used by those skilled in the art to determine the prognosis of MDS in a subject. In one embodiment, the evaluation step is carried out after a treatment period or treatment cycle (*e.g.*, time from administration of first dose) of about 1 week, about 2 weeks, about 3 weeks, about 4 weeks, about 5 weeks, about 6 weeks, about 7, weeks, about 8 weeks, about 9 weeks, about 10 weeks, about 12 weeks, about 14 weeks, about 16 weeks, about 18 weeks, about 20 weeks, about 22 weeks, about 24 weeks, about 26 weeks, about 28 weeks, about 30 weeks, about 32 weeks, about 34 weeks, about 36 weeks, about 38 weeks, about 40 weeks, about 42 weeks, about 44 weeks, about 46 weeks, about 48 weeks, about 50 weeks, or greater than 50 weeks. In one embodiment, the evaluation step may be repeated periodically (e.g., every 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 18, 20, 22, 24, or greater than 24 weeks).

[00171] In certain embodiments, appropriate biomarkers may be used to determine or predict the effect of the methods provided herein on the disease state and to provide guidance

as to the dosing schedule. For example, particular embodiments herein provide a method for determining whether a patient diagnosed with MDS has an increased probability of obtaining a greater benefit from treatment with a pharmaceutical composition comprising a cytidine analog by assessing the patient's nucleic acid methylation status. In particular embodiments, the cytidine analog is 5-azacytidine. In particular embodiments, the nucleic acid is DNA or RNA. In particular embodiments, the greater benefit is an overall survival benefit. In particular embodiments, the methylation status is examined in one or more genes, *e.g.*, genes associated with MDS, or genes described herein elsewhere. Specific embodiments involve methods for determining whether baseline DNA methylation levels influence overall survival in patients with MDS treated with 5-azacytidine. Specific embodiments provide methods for determining whether gene promoter methylation levels influence overall survival in patients with MDS.

[00172] In one embodiment, provided herein is a method for determining whether a patient diagnosed with MDS has an increased probability of obtaining a greater benefit from treatment with a pharmaceutical composition comprising a cytidine analog by assessing the gene expression profile in the patient. In one embodiment, provided herein is a method for determining whether a patient diagnosed with MDS has an increased probability of obtaining a greater benefit from treatment with a pharmaceutical composition comprising a cytidine analog by assessing molecular markers, including one or more cell cycle markers, apoptosis markers, and DNA damage markers. In one embodiment, provided herein is a method for determining whether a patient diagnosed with MDS has increased probability of obtaining a greater benefit from treatment with a pharmaceutical composition comprising a cytidine analog by assessing methylation of one or more gene(s), including, but not limited to, *ABHD14A*, *ABO*, *ADAMTS18*, *ADRA2B*, *ADRB3*, *AIRE*, *AKAP12*, *ALOX15B*, *ALS2CR11*, *AMT*, *ANKRD33*, *APC2*, *AVP*, *BHMT*, *C18orf22*, *C19orf30*, *C1orf172*, *C1orf87*, *C3orf15*, *C1QTNF6*, *C22orf27*, *C7orf16*, *C7orf41*, *CBX7*, *CCDC19*, *CCDC81*, *CD164L2*, *CDH1*, *CDKN2B*, *CHAD*, *CHRNG*, *CIDEB*, *CKMT1B*, *CKMT2*, *CLCN6*, *CLDN6*, *CLDN9*, *CNTN4*, *CPT1B*, *CRHBP*, *CXCL5*, *CYP26C1*, *CYP2E1*, *DES*, *DPYS*, *DYDC1*, *EGFL7*, *ELMO3*, *ENTPD2*, *ENTPD3*, *ESR1*, *EYA4*, *F2RL2*, *FAM57B*, *FBLN1*, *FBXO2*, *FKBP1B*, *FLJ44881*, *FLVCR2*, *FREQ*, *FZD9*, *GAB1*, *GAS2L2*, *GATA4*, *GBGT1*, *GDF5*, *GHSR*, *GNAS*, *GNMT*, *GNPNAT1*, *GP1BA*, *GPR25*, *GRM6*, *GSTM5*, *HCN4*, *HIST1H1A*, *HOXD4*, *HSPA2*, *HTATIP2*, *HTR7*, *HYDIN*, *IGDCC3*, *ILDR1*, *IRF6*, *KAZALD1*, *KCNA6*, *KCNK3*, *KCNQ1*, *KIAA0427*, *KIR3DX1*, *KRT25*, *KRT7*, *KRT72*, *LAD1*, *LAMA4*, *LAMC2*, *LGTN*, *LRRC17*, *LTF*, *MBD3L1*, *MEGF10*, *MICAL1*, *MRPL28*, *MTMR9*, *MTNR1B*, *NALCN*, *NCAN*, *NCOR2*,

NDRG2, *NDUFAF3*, *NEUROG1*, *NGB*, *NPFFR2*, *NPM2*, *NPPB*, *NPR2*, *NXN*, *OBFC2B*, *OGFR*, *ONECUT2*, *OTOP1*, *OXT*, *PACSIN1*, *PAOX*, *PARP3*, *PAX1*, *PCDH8*, *PCDHAC2*, *PDE4C*, *PF4V1*, *PKDREJ*, *PM20D1*, *POMC*, *POU3F1*, *PPAPDC3*, *PRIC285*, *PRLH*, *PSMD11*, *PTGIS*, *RAB36*, *RAP1GAP*, *RASGRF1*, *RASIP1*, *RBPJL*, *RLN1*, *RPL36*, *RPL36AL*, *RPUSD3*, *SCG5*, *SCMH1*, *SCUBE3*, *SEMA3B*, *SGPP2*, *SHROOM1*, *SKAP1*, *SLC12A8*, *SLC5A8*, *SNN*, *SORBS3*, *SPG7*, *SPINT1*, *SRD5A2*, *SRRT*, *SSTR4*, *STMN1*, *TBC1D1*, *TCEA2*, *TCF15*, *TFAP2E*, *TGFBI*, *TIAM1*, *TMEM125*, *TMEM151A*, *TMEM184A*, *TMEM189*, *TMOD3*, *TNNT1*, *TP53INP1*, *TRPC4*, *TRPM3*, *UNC80*, *VAMP5*, *VHL*, *VSTM1*, *WBSCR27*, *WDR52*, *WT1*, *ZFP41*, *ZNF205*, and/or *ZNF710*. In one embodiment, provided herein is a method for determining whether a patient diagnosed with MDS has increased probability of obtaining a greater benefit from treatment with a pharmaceutical composition comprising a cytidine analog by assessing methylation of one or more gene(s), including, but not limited to, WT1. In particular embodiments, the cytidine analog is 5-azacytidine. In particular embodiments, the greater benefit is an overall survival benefit.

5.4.2 Administration of Cytidine Analogs

[00173] Certain methods herein provide administration of the cytidine analog by, *e.g.*, intravenous (IV), subcutaneous (SC) or oral routes administration. Certain embodiments herein provide co-administration of a cytidine analog (*e.g.*, 5-azacytidine) with one or more additional active agents to provide a synergistic therapeutic effect in subjects in need thereof. The co-administered agent(s) may be a cancer therapeutic agent, as described herein. In certain embodiments, the co-administered agent(s) may be dosed, *e.g.*, orally or by injection (*e.g.*, IV or SC).

[00174] Certain embodiments herein provide methods for treating disorders of abnormal cell proliferation comprising administering a cytidine analog using, *e.g.*, IV, SC and/or oral administration methods. In certain embodiments, treatment cycles comprise multiple doses administered to a subject in need thereof over multiple days (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or greater than 14 days), optionally followed by treatment dosing holidays (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, or greater than 28 days). Suitable dosage amounts for the methods provided herein include, *e.g.*, therapeutically effective amounts and prophylactically effective amounts. For example, in certain embodiments, the amount of the cytidine analog (*e.g.*, 5-azacytidine) administered in the methods provided herein may range, *e.g.*, between about 50 mg/m^2/day and about 2,000 mg/m^2/day, between about 100 mg/m^2/day and about 1,000 mg/m^2/day,

between about 100 mg/m^2/day and about 500 mg/m^2/day, between about 50 mg/m^2/day and about 500 mg/m^2/day, between about 50 mg/m^2/day and about 200 mg/m^2/day, between about 50 mg/m^2/day and about 100 mg/m^2/day, between about 50 mg/m^2/day and about 75 mg/m^2/day, or between about 120 mg/m^2/day and about 250 mg/m^2/day. In certain embodiments, particular dosages are, *e.g.*, about 50 mg/m^2/day, about 60 mg/m^2/day, about 75 mg/m^2/day, about 80 mg/m^2/day, about 100 mg/m^2/day, about 120 mg/m^2/day, about 140 mg/m^2/day, about 150 mg/m^2/day, about 180 mg/m^2/day, about 200 mg/m^2/day, about 220 mg/m^2/day, about 240 mg/m^2/day, about 250 mg/m^2/day, about 260 mg/m^2/day, about 280 mg/m^2/day, about 300 mg/ m^2/day, about 320 mg/m^2/day, about 350 mg/m^2/day, about 380 mg/m^2/day, about 400 mg/m^2/day, about 450 mg/m^2/day, or about 500 mg/m^2/day. In certain embodiments, particular dosages are, *e.g.*, up to about 100 mg/m^2/day, up to about 120 mg/m^2/day, up to about 140 mg/m^2/day, up to about 150 mg/m^2/day, up to about 180 mg/m^2/day, up to about 200 mg/m^2/day, up to about 220 mg/m^2/day, up to about 240 mg/m^2/day, up to about 250 mg/m^2/day, up to about 260 mg/m^2/day, up to about 280 mg/m^2/day, up to about 300 mg/ m^2/day, up to about 320 mg/m^2/day, up to about 350 mg/m^2/day, up to about 380 mg/m^2/day, up to about 400 mg/m^2/day, up to about 450 mg/m^2/day, up to about 500 mg/m^2/day, up to about 750 mg/m^2/day, or up to about 1000 mg/m^2/day.

[00175] In one embodiment, the amount of the cytidine analog (*e.g.*, 5-azacytidine) administered in the methods provided herein may range, *e.g.*, between about 5 mg/day and about 2,000 mg/day, between about 10 mg/day and about 2,000 mg/day, between about 20 mg/day and about 2,000 mg/day, between about 50 mg/day and about 1,000 mg/day, between about 100 mg/day and about 1,000 mg/day, between about 100 mg/day and about 500 mg/day, between about 150 mg/day and about 500 mg/day, or between about 150 mg/day and about 250 mg/day. In certain embodiments, particular dosages are, *e.g.*, about 10 mg/day, about 20 mg/day, about 50 mg/day, about 75 mg/day, about 100 mg/day, about 120 mg/day, about 150 mg/day, about 200 mg/day, about 250 mg/day, about 300 mg/day, about 350 mg/day, about 400 mg/day, about 450 mg/day, about 500 mg/day, about 600 mg/day, about 700 mg/day, about 800 mg/day, about 900 mg/day, about 1,000 mg/day, about 1,200 mg/day, or about 1,500 mg/day. In certain embodiments, particular dosages are, *e.g.*, up to about 10 mg/day, up to about 20 mg/day, up to about 50 mg/day, up to about 75 mg/day, up to about 100 mg/day, up to about 120 mg/day, up to about 150 mg/day, up to about 200 mg/day, up to about 250 mg/day, up to about 300 mg/day, up to about 350 mg/day, up to about 400 mg/day, up to about 450 mg/day, up to about 500 mg/day, up to about 600 mg/day, up to about 700

mg/day, up to about 800 mg/day, up to about 900 mg/day, up to about 1,000 mg/day, up to about 1,200 mg/day, or up to about 1,500 mg/day.

[00176] In one embodiment, the amount of the cytidine analog (*e.g.*, 5-azacytidine) in the pharmaceutical composition or dosage form provided herein may range, *e.g.*, between about 5 mg and about 2,000 mg, between about 10 mg and about 2,000 mg, between about 20 mg and about 2,000 mg, between about 50 mg and about 1,000 mg, between about 50 mg and about 500 mg, between about 50 mg and about 250 mg, between about 100 mg and about 500 mg, between about 150 mg and about 500 mg, or between about 150 mg and about 250 mg. In certain embodiments, particular amounts are, *e.g.*, about 10 mg, about 20 mg, about 50 mg, about 75 mg, about 100 mg, about 120 mg, about 150 mg, about 200 mg, about 250 mg, about 300 mg, about 350 mg, about 400 mg, about 450 mg, about 500 mg, about 600 mg, about 700 mg, about 800 mg, about 900 mg, about 1,000 mg, about 1,200 mg, or about 1,500 mg. In certain embodiments, particular amounts are, *e.g.*, up to about 10 mg, up to about 20 mg, up to about 50 mg, up to about 75 mg, up to about 100 mg, up to about 120 mg, up to about 150 mg, up to about 200 mg, up to about 250 mg, up to about 300 mg, up to about 350 mg, up to about 400 mg, up to about 450 mg, up to about 500 mg, up to about 600 mg, up to about 700 mg, up to about 800 mg, up to about 900 mg, up to about 1,000 mg, up to about 1,200 mg, or up to about 1,500 mg.

[00177] In one embodiment, depending on the disease to be treated and the subject's condition, the cytidine analog (*e.g.*, 5-azacytidine) may be administered by oral, parenteral (*e.g.*, intramuscular, intraperitoneal, intravenous, CIV, intracistemal injection or infusion, subcutaneous injection, or implant), inhalation, nasal, vaginal, rectal, sublingual, or topical (*e.g.*, transdermal or local) routes of administration. The cytidine analog may be formulated, alone or together with one or more active agent(s), in suitable dosage unit with pharmaceutically acceptable excipients, carriers, adjuvants and vehicles, appropriate for each route of administration. In one embodiment, the cytidine analog (*e.g.*, 5-azacytidine) is administered orally. In another embodiment, the cytidine analog (*e.g.*, 5-azacytidine) is administered parenterally. In yet another embodiment, the cytidine analog (*e.g.*, 5-azacytidine) is administered intravenously.

[00178] In one embodiment, the cytidine analog (*e.g.*, 5-azacytidine) can be delivered as a single dose such as, *e.g.*, a single bolus injection, or oral tablets or pills; or over time such as, *e.g.*, continuous infusion over time or divided bolus doses over time. In one embodiment, the cytidine analog (*e.g.*, 5-azacytidine) can be administered repetitively if necessary, for example, until the patient experiences stable disease or regression, or until the patient

experiences disease progression or unacceptable toxicity. For example, stable disease for solid tumors generally means that the perpendicular diameter of measurable lesions has not increased by 25% or more from the last measurement. *See, e.g.*, Response Evaluation Criteria in Solid Tumors (RECIST) Guidelines, *Journal of the National Cancer Institute* 92(3): 205-216 (2000). Stable disease or lack thereof is determined by methods known in the art such as evaluation of patient's symptoms, physical examination, visualization of the tumor that has been imaged using X-ray, CAT, PET, or MRI scan and other commonly accepted evaluation modalities.

[00179] In one embodiment, the cytidine analog (*e.g.*, 5-azacytidine) can be administered once daily (QD), or divided into multiple daily doses such as twice daily (BID), three times daily (TID), and four times daily (QID). In one embodiment, the administration can be continuous (*i.e.*, daily for consecutive days or every day), intermittent, *e.g.*, in cycles (*i.e.*, including days, weeks, or months of rest when no drug is administered). In one embodiment, the cytidine analog (*e.g.*, 5-azacytidine) is administered daily, for example, once or more than once each day for a period of time. In one embodiment, the cytidine analog (*e.g.*, 5-azacytidine) is administered daily for an uninterrupted period of at least 7 days, in some embodiments, up to 52 weeks. In one embodiment, the cytidine analog (*e.g.*, 5-azacytidine) is administered intermittently, *i.e.*, stopping and starting at either regular or irregular intervals. In one embodiment, the cytidine analog (*e.g.*, 5-azacytidine) is administered for one to six days per week. In one embodiment, the cytidine analog (*e.g.*, 5-azacytidine) is administered in cycles (*e.g.*, daily administration for two to eight consecutive weeks, then a rest period with no administration for up to one week; or *e.g.*, daily administration for one week, then a rest period with no administration for up to three weeks). In one embodiment, the cytidine analog (*e.g.*, 5-azacytidine) is administered on alternate days. In one embodiment, the cytidine analog (*e.g.*, 5-azacytidine) is administered in cycles (*e.g.*, administered daily or continuously for a certain period interrupted with a rest period).

[00180] In one embodiment, the frequency of administration ranges from about daily to about monthly. In certain embodiments, the cytidine analog (*e.g.*, 5-azacytidine) is administered once a day, twice a day, three times a day, four times a day, once every other day, twice a week, once every week, once every two weeks, once every three weeks, or once every four weeks. In one embodiment, the cytidine analog (*e.g.*, 5-azacytidine) is administered once a day. In another embodiment, the cytidine analog (*e.g.*, 5-azacytidine) is administered twice a day. In yet another embodiment, the cytidine analog (*e.g.*, 5-

azacytidine) is administered three times a day. In still another embodiment, the cytidine analog (*e.g.*, 5-azacytidine) is administered four times a day.

[00181] In one embodiment, the cytidine analog (*e.g.*, 5-azacytidine) is administered once per day from one day to six months, from one week to three months, from one week to four weeks, from one week to three weeks, or from one week to two weeks. In certain embodiments, the cytidine analog (*e.g.*, 5-azacytidine) is administered once per day for one week, two weeks, three weeks, or four weeks. In one embodiment, the cytidine analog (*e.g.*, 5-azacytidine) is administered once per day for one week. In another embodiment, the cytidine analog (*e.g.*, 5-azacytidine) is administered once per day for two weeks. In yet another embodiment, the cytidine analog (*e.g.*, 5-azacytidine) is administered once per day for three weeks. In still another embodiment, the cytidine analog (*e.g.*, 5-azacytidine) is administered once per day for four weeks.

[00182] In one embodiment, the cytidine analog (*e.g.*, 5-azacytidine) is administered once per day for about 1 week, about 2 weeks, about 3 weeks, about 4 weeks, about 6 weeks, about 9 weeks, about 12 weeks, about 15 weeks, about 18 weeks, about 21 weeks, or about 26 weeks. In certain embodiments, the cytidine analog (*e.g.*, 5-azacytidine) is administered intermittently. In certain embodiments, the cytidine analog (*e.g.*, 5-azacytidine) is administered intermittently in the amount of between about 50 mg/m^2/day and about 2,000 mg/m^2/day. In certain embodiments, the cytidine analog (*e.g.*, 5-azacytidine) is administered continuously. In certain embodiments, the cytidine analog (*e.g.*, 5-azacytidine) is administered continuously in the amount of between about 50 mg/m^2/day and about 1,000 mg/m^2/day.

[00183] In certain embodiments, the cytidine analog (*e.g.*, 5-azacytidine) is administered to a patient in cycles (*e.g.*, daily administration for one week, then a rest period with no administration for up to three weeks). Cycling therapy involves the administration of an active agent for a period of time, followed by a rest for a period of time, and repeating this sequential administration. Cycling therapy can reduce the development of resistance, avoid or reduce the side effects, and/or improves the efficacy of the treatment.

[00184] In one embodiment, 5-azacytidine is administered to a patient in cycles. In one embodiment, a method provided herein comprises administering 5-azacytidine in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, or greater than 40 cycles. In one embodiment, the median number of cycles administered in a group of patients is about 1, about 2, about 3, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 11, about 12, about 13, about 14,

about 15, about 16, about 17, about 18, about 19, about 20, about 21, about 22, about 23, about 24, about 25, about 26, about 27, about 28, about 29, about 30, or greater than about 30 cycles.

[00185] In one embodiment, 5-azacytidine is administered to a patient at a dose provided herein over a cycle of 28 days which consists of a 7-day treatment period and a 21-day resting period. In one embodiment, 5-azacytidine is administered to a patient at a dose provided herein each day from day 1 to day 7, followed with a resting period from day 8 to day 28 with no administration of 5-azacytidine. In one embodiment, 5-azacytidine is administered to a patient in cycles, each cycle consisting of a 7-day treatment period followed with a 21-day resting period. In particular embodiments, 5-azacytidine is administered to a patient at a dose of about 50, about 60, about 70, about 75, about 80, about 90, or about 100 $mg/m^2/d$, for 7 days, followed with a resting period of 21 days. In one embodiment, 5-azacytidine is administered intravenously. In one embodiment, 5-azacytidine is administered subcutaneously.

[00186] In other embodiments, 5-azacytidine is administered orally in cycles.

[00187] Accordingly, in one embodiment, the cytidine analog (*e.g.*, 5-azacytidine) is administered daily in single or divided doses for about one week, about two weeks, about three weeks, about four weeks, about five weeks, about six weeks, about eight weeks, about ten weeks, about fifteen weeks, or about twenty weeks, followed by a rest period of about 1 day to about ten weeks. In one embodiment, the methods provided herein contemplate cycling treatments of about one week, about two weeks, about three weeks, about four weeks, about five weeks, about six weeks, about eight weeks, about ten weeks, about fifteen weeks, or about twenty weeks. In some embodiments, the cytidine analog (*e.g.*, 5-azacytidine) is administered daily in single or divided doses for about one week, about two weeks, about three weeks, about four weeks, about five weeks, or about six weeks with a rest period of about 1, 3, 5, 7, 9, 12, 14, 16, 18, 20, 22, 24, 26, 28, 29, or 30 days. In some embodiments, the rest period is 1 day. In some embodiments, the rest period is 3 days. In some embodiments, the rest period is 7 days. In some embodiments, the rest period is 14 days. In some embodiments, the rest period is 28 days. The frequency, number and length of dosing cycles can be increased or decreased.

[00188] In one embodiment, the methods provided herein comprise: i) administering to the subject a first daily dose of the cytidine analog (*e.g.*, 5-azacytidine); ii) optionally resting for a period of at least one day where the cytidine analog (*e.g.*, 5-azacytidine) is not administered to the subject; iii) administering a second dose of the cytidine analog (*e.g.*, 5-azacytidine) to

the subject; and iv) repeating steps ii) to iii) a plurality of times. In certain embodiments, the first daily dose is between about 50 mg/m^2/day and about 2,000 mg/m^2/day. In certain embodiments, the second daily dose is between about 50 mg/m^2/day and about 2,000 mg/m^2/day. In certain embodiments, the first daily dose is higher than the second daily dose. In certain embodiments, the second daily dose is higher than the first daily dose. In one embodiment, the rest period is 2 days, 3 days, 5 days, 7 days, 10 days, 12 days, 13 days, 14 days, 15 days, 17 days, 21 days, or 28 days. In one embodiment, the rest period is at least 2 days and steps ii) through iii) are repeated at least three times. In one embodiment, the rest period is at least 2 days and steps ii) through iii) are repeated at least five times. In one embodiment, the rest period is at least 3 days and steps ii) through iii) are repeated at least three times. In one embodiment, the rest period is at least 3 days and steps ii) through iii) are repeated at least five times. In one embodiment, the rest period is at least 7 days and steps ii) through iii) are repeated at least three times. In one embodiment, the rest period is at least 7 days and steps ii) through iii) are repeated at least five times. In one embodiment, the rest period is at least 14 days and steps ii) through iii) are repeated at least three times. In one embodiment, the rest period is at least 14 days and steps ii) through iii) are repeated at least five times. In one embodiment, the rest period is at least 21 days and steps ii) through iii) are repeated at least three times. In one embodiment, the rest period is at least 21 days and steps ii) through iii) are repeated at least five times. In one embodiment, the rest period is at least 28 days and steps ii) through iii) are repeated at least three times. In one embodiment, the rest period is at least 28 days and steps ii) through iii) are repeated at least five times. In one embodiment, the methods provided herein comprise: i) administering to the subject a first daily dose of the cytidine analog (*e.g.*, 5-azacytidine) for 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14 days; ii) resting for a period of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, or 28 days; iii) administering to the subject a second daily dose of the cytidine analog (*e.g.*, 5-azacytidine) for 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14 days; and iv) repeating steps ii) to iii) a plurality of times. In one embodiment, the methods provided herein comprise: i) administering to the subject a daily dose of the cytidine analog (*e.g.*, 5-azacytidine) for 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14 days; ii) resting for a period of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, or 28 days; and iii) repeating steps i) to ii) a plurality of times. In one embodiment, the methods provided herein comprise: i) administering to the subject a daily dose of the cytidine analog (*e.g.*, 5-azacytidine) for 7 days; ii) resting for a period of 21 days; and iii) repeating steps i) to ii) a plurality of times. In one embodiment, the daily dose is between

about 50 mg/m^2/day and about 2,000 mg/m^2/day. In one embodiment, the daily dose is between about 50 mg/m^2/day and about 1,000 mg/m^2/day. In one embodiment, the daily dose is between about 50 mg/m^2/day and about 500 mg/m^2/day. In one embodiment, the daily dose is between about 50 mg/m^2/day and about 200 mg/m^2/day. In one embodiment, the daily dose is between about 50 mg/m^2/day and about 100 mg/m^2/day.

[00189] In certain embodiments, the cytidine analog (*e.g.*, 5-azacytidine) is administered continuously for between about 1 and about 52 weeks. In certain embodiments, the cytidine analog (*e.g.*, 5-azacytidine) is administered continuously for about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 months. In certain embodiments, the cytidine analog (*e.g.*, 5-azacytidine) is administered continuously for about 14, about 28, about 42, about 84, or about 112 days. It is understood that the duration of the treatment may vary with the age, weight, and condition of the subject being treated, and may be determined empirically using known testing protocols or according to the professional judgment of the person providing or supervising the treatment. The skilled clinician will be able to readily determine, without undue experimentation, an effective drug dose and treatment duration, for treating an individual subject having a particular type of cancer.

5.4.3 Co-Administered Therapeutic Agents

[00190] In certain embodiments, methods provided herein for treating a cancer or an MDS, comprise co-administering a cytidine analog, such as, for example, 5-azacytidine, with one or more therapeutic agents, such as, for example, cancer therapeutic agents, to yield a synergistic therapeutic effect. The co-administered therapeutic agents include, but are not limited to, *e.g.*, cytotoxic agents, anti-metabolites, antifolates, HDAC inhibitors such as MGCD0103 (a.k.a. *N*-(2-aminophenyl)-4-((4-(pyridin-3-yl)pyrimidin-2-ylamino)methyl)benzamide), DNA intercalating agents, DNA cross-linking agents, DNA alkylating agents, DNA cleaving agents, topoisomerase inhibitors, CDK inhibitors, JAK inhibitors, anti-angiogenic agents, Bcr-Abl inhibitors, HER2 inhibitors, EGFR inhibitors, VEGFR inhibitors, PDGFR inhibitors, HGFR inhibitors, IGFR inhibitors, c-Kit inhibitors, Ras pathway inhibitors, PI3K inhibitors, multi-targeted kinase inhibitors, mTOR inhibitors, anti-estrogens, anti-androgens, aromatase inhibitors, somatostatin analogs, ER modulators, anti-tubulin agents, vinca alkaloids, taxanes, HSP inhibitors, Smoothened antagonists, telomerase inhibitors, COX-2 inhibitors, anti-metastatic agents, immunosuppressants, biologics such as antibodies, and hormonal therapies. In particular embodiment, the co-

administered therapeutic agent is thalidomide, lenalidomide, or pomalidomide. The co-administered agent may be dosed, *e.g.*, orally or by injection.

[00191] In one embodiment, the route of the administration of the cytidine analog (*e.g.*, 5-azacytidine) is independent of the route of the administration of a second therapy. In one embodiment, the cytidine analog (*e.g.*, 5-azacytidine) is administered orally. In another embodiment, the cytidine analog (*e.g.*, 5-azacytidine) is administered intravenously. In accordance with these embodiments, the cytidine analog (*e.g.*, 5-azacytidine) is administered orally or intravenously, and the second therapy can be administered orally, parenterally, intraperitoneally, intravenously, intraarterially, transdermally, sublingually, intramuscularly, rectally, transbuccally, intranasally, liposomally, via inhalation, vaginally, intraoccularly, via local delivery by catheter or stent, subcutaneously, intraadiposally, intraarticularly, intrathecally, or in a slow release dosage form. In one embodiment, the cytidine analog (*e.g.*, 5-azacytidine) and a second therapy are administered by the same mode of administration, *e.g.*, orally or intravenously. In another embodiment, the cytidine analog (*e.g.*, 5-azacytidine) is administered by one mode of administration, *e.g.*, intravenously, whereas the second agent (*e.g.*, an anticancer agent) is administered by another mode of administration, *e.g.*, orally. In another embodiment, the cytidine analog (*e.g.*, 5-azacytidine) is administered by one mode of administration, *e.g.*, orally, whereas the second agent (*e.g.*, an anticancer agent) is administered by another mode of administration, *e.g.*, intravenously.

[00192] In one embodiment, each method provided herein may independently, further comprise the step of administering a second therapeutic agent. In one embodiment, the second therapeutic agent is an anticancer agent. In one embodiment, the anticancer agent is an antimetabolite, including, but not limited to, 5-fluoro uracil, methotrexate, cytarabine, high dose cytarabine, and fludarabine. In one embodiment, the anticancer agent is an antimicrotubule agent, including, but not limited to, vinca alkaloids (*e.g.*, vincristine and vinblastine) and taxanes (*e.g.*, paclitaxel and docetaxel). In one embodiment, the anticancer agent is an alkylating agent, including, but not limited to, cyclophosphamide, melphalan, carmustine, and nitrosoureas (*e.g.*, hydroxyurea and bischloroethylnitrosurea). In one embodiment, the anticancer agent is a platinum agent, including, but not limited to, cisplatin, carboplatin, oxaliplatin, satraplatin (JM-216), and CI-973. In one embodiment, the anticancer agent is an anthracycline, including, but not limited to, doxrubicin and daunorubicin. In one embodiment, the anticancer agent is an antitumor antibiotic, including, but not limited to, mitomycin, idarubicin, adriamycin, and daunomycin (also known as daunorubicin). In one embodiment, the anticancer agent is a topoisomerase inhibitor, *e.g.*, etoposide and

camptothecins. In one embodiment, the anticancer agent is selected from the group consisting of adriamycin, busulfan, cytarabine, cyclophosphamide, dexamethasone, fludarabine, fluorouracil, hydroxyurea, interferons, oblimersen, platinum derivatives, taxol, topotecan, and vincristine.

[00193] In one embodiment, other therapies or anticancer agents that may be used in combination with the cytidine analog (*e.g.*, 5-azacytidine) include surgery, radiotherapy (*e.g.*, gamma-radiation, neutron beam radiotherapy, electron beam radiotherapy, proton therapy, brachytherapy, and systemic radioactive isotopes), endocrine therapy, biologic response modifiers (*e.g.*, interferons, interleukins, and tumor necrosis factor (TNF)), hyperthermia and cryotherapy, agents to attenuate any adverse effects (*e.g.*, antiemetics), and other approved chemotherapeutic drugs, including, but not limited to, alkylating drugs (mechlorethamine, chlorambucil, cyclophosphamide, melphalan, and ifosfamide), antimetabolites (cytarabine, high dose cytarabine, and methotrexate), purine antagonists and pyrimidine antagonists (6-mercaptopurine, 5-fluorouracil, cytarabine, and gemcitabine), spindle poisons (vinblastine, vincristine, vinorelbine, and paclitaxel), podophyllotoxins (etoposide, irinotecan, and topotecan), antibiotics (daunorubicin, doxorubicin, bleomycin, and mitomycin), nitrosoureas (carmustine and lomustine), inorganic ions (cisplatin and carboplatin), enzymes (asparaginase), and hormones (tamoxifen, leuprolide, flutamide, and megestrol), imatinib, adriamycin, dexamethasone, and cyclophosphamide. For additional available cancer therapies, see, *e.g,* http://www.nci.nih.gov/; for a list of FDA approved oncology drugs, see, *e.g.,* http://www.fda.gov/, The Merck Manual, 18th Ed. 2006, and PDR: Physician Desk Reference 2010, 64th Ed. 2009; the contents of each of which are hereby incorporated by reference in their entireties.

[00194] In one embodiment, provided herein is a kit incorporating a biomarker provided herein. In some embodiments, the kit is a diagnostic kit. For example, single gene epigenetics biomarkers may be used in the diagnosis of certain cancers. *See, e.g.*, www.epigenomics.com. Such gene signature may be developed into a diagnostic kit, for use in a high-throughput methylation assay. In other embodiments, employing gene array technology, customized gene chip for a particular gene signature or pattern may be used in a kit provided herein. *See, e.g.,* www.pathworkdx.com.

6. EXAMPLES

[00195] The following examples are provided by way of illustration, not limitation.

6.1 Example 1

[00196] This phase III randomized trial assessed the effect of 5-azacytidine on prolonging overall survival in patients with higher risk MDS compared with 3 other frequently used conventional care regimens.

[00197] A phase III, international, multi-center, prospective, randomized, controlled, parallel group trial was conducted and demonstrated prolonged overall survival in higher risk MDS patients as compared to conventional care regimens and best supportive care. (This study is referred to herein as the "AZA-001" study). *See, e.g.*, Fenaux *et al.*, *Lancet Oncology*, 2009, 10:223–32. The primary study objective and endpoint were overall survival (OS), comparing 5-azacytidine and conventional care regimens. Secondary objectives and endpoints included time to transformation to acute myeloid leukemia (AML), red blood cell transfusion independence, hematologic responses and improvement, infections requiring IV therapy, and safety.

[00198] Eligible patients were 18 years or older with higher risk MDS, defined as an IPSS of Intermediate-2 or High and FAB-defined RAEB, RAEB-T, or non-myeloproliferative chronic myelomonocytic leukemia (CMML), using modified FAB criteria (blood monocytes greater than 1 x 10^9/L, dysplasia in 1 or more myeloid cell lines, 10%-29% marrow blasts, and a white blood count below 13 x 10^9/L). Patients were to have an Eastern Cooperative Oncology Group (ECOG) performance status of 0-2 and life expectancy of 3 months or more. Patients with secondary therapy-related MDS, prior 5-azacytidine treatment, or eligibility for allogeneic stem cell transplantation were excluded.

[00199] The Phase III, international, multi-center, randomized, controlled, parallel-group trial was conducted in accordance with the Declaration of Helsinki. All patients provided written informed consent, and the study was approved by the institutional review boards at all participating study sites. Enrollment to the trial and monitoring was conducted by site investigators and central pathology reviewers with standardized central review of cytogenetic data. An independent Data Safety Monitoring Board reviewed safety data and conducted blinded review of a scheduled interim analysis.

[00200] Patients were randomized to 1 of 2 treatment groups: 5-azacytidine plus best supportive care (BSC) or conventional care regimens (CCR) plus BSC. Patients were randomized 1:1 to receive 5-azacytidine or CCR. Prior to randomization, investigators pre-selected (based on age, health and disease status, co-morbidities, etc.) the most appropriate one of three conventional CCR groups for higher risk MDS patients, which the patients then

received if randomized to CCR. Patients randomized to 5-azacytidine received 5-azacytidine regardless of CCR selection. This pre-randomization step was performed to enable meaningful comparisons of CCR subgroups with relevant 5-azacytidine-treated subgroups. No crossover was allowed in this trial and administration of erythropoietin or darbepoetin was prohibited. Balanced enrollment across treatments was ensured using blocked randomization with patients stratified by FAB subtype and IPSS risk group.

[00201] During the treatment phase of the trial, all regimens were continued until study end or patient discontinuation due to relapse, disease progression, unacceptable toxicity, or transformation to AML (defined as 30% or greater bone marrow blasts). 5-Azacytidine was administered subcutaneously at 75 mg/m^2/day for 7 days every 28 days (delayed as needed until cell line recovery), which constituted one cycle of therapy, for at least 6 cycles until study end unless treatment was discontinued due to unacceptable toxicity, relapse after response, or disease progression. The CCR group consisted of 3 treatment regimens administered until study end or treatment discontinuation: BSC only (including blood product transfusions, antibiotics, with G-CSF for neutropenic infection); low-dose ara-C (LDara-C): 20 mg/m^2/day subcutaneously for 14 days, every 28-42 days (delayed as needed until cell line recovery) for at least 4 cycles; or intensive chemotherapy, *i.e.* induction with ara-C 100-200 mg/m^2/day by continuous intravenous infusion for 7 days plus 3 days of intravenous daunorubicin (45-60 mg/m^2/day), idarubicin (9-12 mg/m^2/day), or mitoxantrone (8-12 mg/m^2/day). Patients with complete or partial remission after induction (defined by IWG criteria for AML, *see e.g.*, *J. Clin. Oncol.* 2003, 21(24):4642-9) received 1-2 consolidation courses with reduced doses of the cytotoxic agents used for induction, followed by BSC only. All patients could receive BSC as needed. After treatment discontinuation, all patients were followed until death or end of study (12 months following randomization of the last patient). Figure 1 shows the study design.

[00202] All efficacy analyses used the intent-to-treat (ITT) population. Safety analyses were performed on the safety population (all patients who received at least 1 dose of study drug and 1 or more post-dose safety assessments). The primary trial endpoint was overall survival (time from randomization until death from any cause), analyzed for the ITT group comparing the 5-azacytidine group and the CCR group, and for predefined subgroups based on age, gender, FAB, IPSS (Int-2, high), IPSS cytogenetics (good, intermediate, and poor) and -7/del(7q) cytogenetic abnormality, IPSS cytopenias (0/1 and 2/3), WHO classification, karyotype, and lactic dehydrogenase (LDH). The primary assessment of overall survival used the ITT population and compared 5-azacytidine with the combined CCR group. A

secondary analysis compared overall survival of 5-azacytidine subgroups (the 3 CCR subgroups of patients who were randomized to 5-azacytidine) with the corresponding CCR subgroups (patients in the corresponding CCR subgroups, who were randomized to CCR).

[00203] Secondary efficacy endpoints were transformation to AML (from randomization until AML transformation [30% bone marrow blast count or greater]), hematologic response and improvement assessed using IWG 2000 criteria for MDS (*See e.g.*, Cheson *et al.*, *Blood* 2000, 96(12):3671-4), red blood cell (RBC) transfusion independence (absence of transfusions during 56 consecutive days), infections requiring intravenous antimicrobials (analyzed from randomization to 28 days post last study visit), and adverse events. Bone marrow samples were collected every 16 weeks during active treatment and as clinically indicated during follow-up. Infections requiring intravenous antimicrobials were counted from randomization to last study visit. Adverse events were assessed using the National Cancer Institute's Common Toxicity Criteria, Version 2.0.

[00204] Time to event was studied using the Kaplan-Meier method; treatment comparisons were made using stratified log-rank tests and Cox proportional-hazards models. All statistical tests were two-sided without correction for multiple testing.

[00205] Efficacy analyses included all patients randomized according to the ITT principle. Overall survival was defined as the time from randomization until death from any cause. Patients for whom death was not observed were censored at the time of last follow-up. Time to transformation to AML was measured from randomization to development of 30% or greater bone marrow blasts. Patients for whom AML transformation was not observed were censored at the time of last adequate bone marrow sample. Randomization and analyses were stratified on FAB subtype and IPSS risk group. Time-to-event curves were estimated according to the Kaplan-Meier method (*See e.g.*, Kaplan *et al.*, J. Am. Stat. Assoc. 1958, 53;457-81) and compared using stratified log-rank tests (primary analysis). Stratified Cox proportional hazards regression models (*See e.g.*, Cox, J. Royal Stat. Soc. B, 1972, 34;184-92) were used to estimate hazard ratios and associated 95% confidence intervals (CI). The primary analysis of overall survival between the 5-azacytidine and combined CCR groups used the stratified Cox proportional hazards model without any covariate adjustments to estimate the hazard ratio. Cox proportional hazards regression with stepwise selection was used to assess the baseline variables of sex, age, time since original MDS diagnosis, ECOG performance status, number of RBC transfusions, number of platelet transfusions, hemoglobin, platelets, absolute neutrophil count, LDH, bone marrow blast percentage, and presence or absence of cytogenetic -7/del(7q) abnormality. The final model included ECOG

performance status, LDH, hemoglobin, number of RBC transfusions and presence or absence of cytogenetic -7/del(7q) abnormality. Secondary analyses used the final Cox proportional hazards model. The consistency of treatment effect across subgroups was assessed by the difference in likelihood ratio between the full model with treatment, subgroup and treatment-by-subgroup interaction, and the reduced model without the interaction.

[00206] Response rates (overall response, transfusion independence, and hematological improvement) were compared between the 5-azacytidine and CCR groups using Fisher's exact test. The rate of infection requiring intravenous antimicrobials was computed as the number of observed infections requiring intravenous antimicrobials divided by the total number of patient-years of follow-up. The relative risk was computed by dividing the 5-azacytidine rate by the CCR rate. The relative risks across the 4 strata were tested for homogeneity using the Breslow-Day test (*See e.g.*, Breslow *et al.*, Chapter 3: Comparisons Among Exposure Groups. In: Heseltine E. ed. *Statistical Methods in Cancer Research* Volume II - The Design and Analysis of Cohort Studies. Lyon: IARC Scientific Publications; 1987:82-119). The Mantel-Haenszel estimate of the common relative risk, the associated 95% CI, and the test that it equals unity were computed (*See e.g.*, Mantel, Cancer Chemotherapy Reports, 1966, 50(3):163-70). This study was designed with 90% power – based on a log rank analysis – to detect a hazard ratio of 0.60 for overall survival in the 5-azacytidine group compared with the CCR group with a two-sided alpha of 0.05. The protocol specified that approximately 354 patients were to be randomized over 18 months and then monitored for at least 12 months of treatment and follow-up, resulting in at least 167 deaths over the 30 month trial period. Recruitment, however, necessitated a longer study period that lasted 42 months with 195 deaths that resulted in a 95% power under the design assumptions of the study. The interim analysis was conducted using an O'Brien-Fleming monitoring boundary and Lan-DeMets alpha spending function to control the overall alpha at 0.05 (*See e.g.*, Lan *et al.*, *Biometrika* 1983, 70(3):659-63).

[00207] 358 Patients (ITT population, 98% Caucasian, 70% male) at 79 sites were randomized: 179 to 5-azacytidine and 179 to CCR (105 to BSC 59%, 49 to LDara-C 27%, and 25 to intensive chemotherapy 14%). Median age was 69 years (range: 38-88) with 258 (72%) patients aged 65 years or older. Baseline demographic and disease characteristics were well balanced between the 5-azacytidine and CCR combined and between 5-azacytidine and the 3 CCR regimens (Table 2A and 2B). As expected, patients in the intensive chemotherapy group were younger. At baseline, 95% of patients were higher risk: RAEB (58%), RAEB-T (34%), CMML (3%), and other (5%). By IPSS, 87% were higher risk: Int-

2 (41%), High (47%), and 13% indeterminate/other. Additionally, 32% of patients were classified as WHO AML (marrow blast count, 20%-30%). Upon IRC review, 10 and 5 patients, respectively, in the 5-azacytidine and CCR groups had received prior radiation, chemotherapy, or cytotoxic therapies for non-MDS conditions, which constituted protocol deviations. 5-Azacytidine was administered for a median of 9 cycles (range 1 to 39) with 86% of patients remaining on the 75 mg/m^2/day dose throughout the study with no adjustments. The median 5-azacytidine cycle length was 34 days (range 15 to 92). LDara-C was administrated for a median of 4.5 cycles (range 1 to 15), BSC only patients for a median of 7 cycles (range 1 to 26, 6.2 months), and intensive chemotherapy for 1 cycle (range 1 to 3, i.e. induction plus 1 or 2 consolidation cycles, with cytarabine and anthracycline). Median follow-up for the overall survival analysis was 21.1 months. Overall analysis (ITT): AZA (N=179 vs. CCR (N=179). Analysis by CCR treatment selection: AZA (N=117) vs. BSC (n=105); AZA (N=45) vs. LD Ara-C (N=49); AZA (N=17) vs. Intensive Chemo (N=25). Four patients in the 5-azacytidine group and 14 in the CCR group never received but were followed for overall survival and were included in the ITT analysis. Eight patients went on to transplant after treatment (4 in the 5-azacytidine group and 4 in the CCR group: BSC [n=2], LDara-C [n=1], intensive chemotherapy [n=1]) and were also included in the ITT analysis.

Overall Survival

[00208] 5-Azacytidine demonstrated statistically superior overall survival vs. conventional care regimens. After a median follow-up of 21.1 months (range 0 to 38.4), median Kaplan-Meier overall survival was 24.4 months in the 5-azacytidine group compared with 15 months in the CCR group, for a difference of 9.4 months (stratified log-rank p=0.0001) (Figure 2). The hazard ratio (Cox Model) was 0.58 (95% CI: 0.43-0.77) indicating a 42% reduction in risk of death in the 5-azacytidine group and a 74% overall survival advantage. At two year, 50.8% (95% CI: 42.1-58.8) of patients in the 5-azacytidine group were alive compared with 26% (95% CI: 18.7-34.3) in the CCR group ($p<0.0001$). After approximately 100 days (about 3 months), with 78% (140/179) of 5-azacytidine patients completing 3 cycles of therapy, the Kaplan-Meier curves for the 5-azacytidine and CCR groups separated for the remainder of the trial.

[00209] Results in the predefined patient subgroups (based on age, gender, FAB classification, IPSS, WHO classification, karyotype, and LDH) also showed a consistent overall survival benefit for the 5-azacytidine group (Figure 2). In particular, IPSS cytogenetic subgroups showed significant overall survival differences favoring the 5-azacytidine group versus the CCR group (hazard ratio; log-rank p): Poor, 11.2 months (0.52,

p=0.011); Intermediate, 9.3 months (0.43, p=0.017); and Good, median not reached (0.62, log-rank p=0.038). In patients with -7/del(7q), median Kaplan-Meier overall survival was 13.1 months (95% CI, 9.9 to 24.5) in the 5-azacytidine group (n=30) compared with 4.6 months (95% CI, 3.5 to 6.7) in the CCR group (n=27) (stratified log-rank p=0.002, hazard ratio, 0.33 (95% CI, 0.16 to 0.68). Additionally, sensitivity analyses exploring the influence of the 8 transplanted patients included in the ITT analyses above did not influence the significance of the overall survival results for 5-azacytidine.

[00210] The survival benefits of 5-azacytidine were consistent regardless of the CCR treatment options. Differences in median overall survival (hazard ratio; log-rank p) between the 5-azacytidine subgroups and the CCR subgroups of BSC, LDara-C, and intensive chemotherapy were 9.6 months (0.58; p=0.005), 9.2 months (0.36; p=0.0006), and 9.4 months (0.76, 95% CI: 0.33 to 1.74), respectively. Similar to the primary overall survival comparison (5-azacytidine vs. CCR), results from the investigator pre-selection subgroup analysis of overall survival showed significant differences between 5-azacytidine (n=117) and BSC (n=105) (p=0.005) and 5-azacytidine (n=45) and LDara-C (n=49) (p=0.0006). The difference in the comparison between 5-azacytidine (n=17) and intensive chemotherapy (n=25), however, was not significant (0.51).

[00211] The significant prolongation of overall survival observed with 5-azacytidine compared with CCR was not dependent on the achievement of complete remission (HR=0.39 [95% CI: 0.14–1.15], log rank p = 0.078). The achievement of hematologic improvement, partial remission, or complete remission contributed to but was not required for improvement in overall survival with 5-azacytidine treatment.

[00212] To date, 5-azacytidine is the only agent to demonstrate survival benefit in MDS compared to conventional care regimens, and the only epigenetic modifier to show survival benefits in cancer. The study described herein represented the largest study ever conducted in higher risk MDS. These results, showing a significant improvement in survival in the most advanced MDS patients, demonstrated the benefit 5-azacytidine can provide to treat the disease. Building on the established data from earlier clinical studies, which showed that 5-azacytidine offers transfusion independence benefits to patients with MDS to improve the overall quality of life, the present study showed that 5-azacytidine not only improves patient's life, but extends it as well.

Secondary Efficacy Endpoints

[00213] Red blood cell transfusion independence, hematologic remission, and hematologic improvement were also significantly increased with 5-azacytidine as compared with combined conventional care regimens. 5-Azacytidine was well tolerated.

Time to AML Transformation

[00214] Assessed over the entire trial, median time to transformation to AML or death was 13.0 months (95% CI: 9.9-15.0) in the 5-azacytidine group compared with 7.6 months (95% CI: 5.4-9.8) in the CCR group (hazard ratio: 0.68, log-rank $p<0.003$).

[00215] Time to AML transformation was assessed during treatment with a median of 26.1 months (95% CI: 15.0-28.7) in the 5-azacytidine group compared with 12.4 months (95% CI: 10.4-15.4) in the CCR group (log-rank $p=0.004$, Figure 3).

[00216] Median time to AML transformation was 17.8 months (95% CI, 13.6 to 23.6) in the 5-azacytidine group compared with 11.5 months (95% CI, 8.3 to 14.5) in the CCR group (hazard ratio, 0.50 (95% CI, 0.35 to 0.70), log rank $p<0.0001$).

Hematologic Response and Improvement Rates

[00217] Complete and partial remission rates were significantly higher in the 5-azacytidine group than in the CCR group. Using the investigator pre-selection analysis, remission rates were generally significantly higher with 5-azacytidine compared with either BSC or LDara-C, but no significant differences in remission rates were observed when comparing 5-azacytidine with intensive chemotherapy. Time to disease progression, relapse after complete or partial remission, or death was significantly longer in the 5-azacytidine group (median, 14.1 months) than in the CCR group (median, 8.8 months, log-rank $P=0.047$). Erythroid and platelet improvement rates were significantly higher in the 5-azacytidine group compared with the CCR group. Major erythroid improvement was observed in 39.5% (62 of 157) vs. 10.6% (17 of 160) of patients in the 5-azacytidine vs. CCR groups, respectively, ($p<0.0001$). Major platelet improvement was observed in 32.6% (46 of 141) vs. 14% (18 of 129) of patients in the 5-azacytidine vs. CCR groups, respectively ($p=0.0003$). No significant differences for major neutrophil improvement were observed between groups. Duration of hematologic improvement was significantly longer in the 5-azacytidine group (median, 13.6 months, 95% CI, 10.1 to 16.3) than in the CCR group (median, 5.2 months, 95% CI, 4.1 to 9.7, $P=0.0002$). 50 of 111 (45%, 95% CI, 35.6 to 54.8) baseline RBC transfusion-dependent patients in the 5-azacytidine group became transfusion independent compared with 13 of 114 (11.4%, 95% CI, 6.2 to 18.7) in the CCR group ($P<0.0001$).

[00218] Overall, 51 of 179 (28.5%) patients in the 5-azacytidine group achieved complete + partial remission compared with 21 of 179 (11.7%, p=0.0001) in the CCR group, including 5 of 105 (5%), 6 of 49 (12.2%), and 10 of 25 (40%) in the BSC, LDara-C, and intensive chemotherapy subgroups, respectively. 17% (30 of 179) and 8% (14 of 179) of patients in the 5-azacytidine and CCR groups, respectively, had a complete remission (p=0.02). The proportion of patients showing any hematologic improvement was significantly higher in the 5-azacytidine group (87 of 177, 49.2%) compared with the CCR group (51 of 178, 28.7%, p<0.0001).

Transfusion Independence

[00219] 45% (95% CI: 35.6-54.8) of patients in the 5-azacytidine group became RBC transfusion independent after being baseline dependent compared with 11.4% (95% CI: 6.2-18.7) in the CCR group (p=0.0001). The effect on platelet transfusions showed no significant differences between the 5-azacytidine and CCR groups, which was likely due to the small numbers of patients with baseline platelet transfusion dependence in the 5-azacytidine (n=38) and CCR (n=27) groups.

Infections Requiring Intravenous Antimicrobials

[00220] The rate of infections requiring intravenous antimicrobials per patient year in the 5-azacytidine group was 0.60 (95% CI, 0.49 to 0.73) compared with 0.92 (95% CI, 0.74 to 1.13) in the CCR group, indicating a 34% reduction (hazard ratio, 0.66, 95% CI, 0.49 to 0.87, P=0.003). Using the investigator pre-selection analysis, per patient year rates were similar when comparing 5-azacytidine (0.66) and BSC (0.61) (hazard ratio: 1.1, 95% CI, 0.74 to 1.65, P=0.68), but significantly lower with 5-azacytidine (0.44) compared with LDara-C (1.00) (hazard ratio: 0.44, 95% CI, 0.25 to 0.86, P=0.017) or with 5-azacytidine (0.64) versus intensive chemotherapy (2.30) (hazard ratio: 0.28, 95% CI, 0.13 to 0.60, P=0.0006).

Safety

[00221] Discontinuations prior to study closure due to adverse events were observed in 12.6% of patients in the 5-azacytidine group compared with (7.3%) in the CCR group. The 2 active therapies in the CCR group showed similar rates with 5-azacytidine but BSC had a much lower rate of discontinuations due to adverse events (3.9%). The most frequently observed treatment-related adverse events (including Grade 3-4 events) were peripheral blood cytopenias, frequently observed across all treatments, which led to discontinuation prior to study closure in 4.6% in the 5-azacytidine group and 2.4% in the CCR group. The most common treatment-related non-hematologic adverse events included injection site reactions with 5-azacytidine, and nausea, vomiting, fatigue, and diarrhea with 5-azacytidine, LDara-C,

and intensive chemotherapy. During the first 3 cycles of treatment, deaths occurred in 14 (8%) of patients in the 5-azacytidine group and 25 (14%) in the CCR group. The most common causes of death in either group were related to underlying disease, thrombocytopenia, sepsis/infection, hemorrhage, and respiratory complications. Transformation to AML was also a cause of death during the first 3 cycles of treatment but observed only in the CCR group. Deaths considered to be related to treatment during the first 3 cycles were observed in 4 patients in the 5-azacytidine group (septic shock, cerebral hemorrhage, hematemesis, respiratory tract infection) and 1 patient in the CCR group (receiving LDara-C) (cerebral ischemia).

[00222] In the higher risk MDS population, the most frequently observed treatment-related adverse events (including Grade 3 and 4 events) were blood cytopenias, frequently observed across all treatments, which led to early withdrawal in 4.6%, 4.5%, and 2% of patients in the 5-azacytidine, LDara-C, and BSC treatment groups, respectively.

[00223] The most common treatment-related non-hematologic adverse events included injection site reactions with 5-azacytidine, and nausea, vomiting, fatigue, and diarrhea across the 5-azacytidine, low-dose ara-C, and intensive chemotherapy treatment groups. During treatment and follow-up, deaths were reported in 45% of patients in the 5-azacytidine group, and 62%, 59%, and 79% of patients, respectively, in the BSC, LDara-C, and intensive chemotherapy subgroups. The major causes of death were infection and AML (>30% blasts).

Discussion:

[00224] Results of the phase III, randomized, controlled comparative trial showed that 5-azacytidine was the first drug treatment to prolong overall survival in higher risk MDS patients. While allogeneic stem cell transplantation is potentially curative in MDS, its use is limited by older age, a lack of donors, and increased transplant-related mortality. In a previous randomized phase III CALGB trial comparing 5-azacytidine with BSC (*See, e.g.*, J. *Clin. Oncol*. 2002, 20(10):2429-40), the 5-azacytidine group showed a trend for improved overall survival over BSC. The finding was possibly limited by a heterogeneous patient population and a cross-over trial design, with 51% of BSC patients subsequently receiving 5-azacytidine. Findings of the CALGB trial were also lessened by the use of BSC, a treatment not considered as intensive care in higher risk MDS by many clinicians.

[00225] No crossover was allowed in the present study. The present study included only patients with higher risk MDS. Additionally, the study compared azacytidine to three frequently used treatments (LDara-C, intensive chemotherapy, or BSC) for higher risk MDS including two active therapies (LDara-C, or intensive chemotherapy). As there is no current

consensus on the use of those three regimens, their allocation for patients was made by the investigators based on patient age, general condition, presence of co-morbidities, and personal choice.

[00226] Overall survival in the present study showed an advantage of 9.4 months for the 5-azacytidine group over the CCR group, corresponding to a 42% reduction in risk of death. The robustness of this overall survival benefit was further shown in the nearly 2-fold higher proportion of patients in the 5-azacytidine group surviving at two years compared with those in the CCR group. This overall survival advantage with 5-azacytidine in the primary, ITT analysis was highly similar to that seen using the secondary, investigator-selection analysis with median survival differences ranging from 9.2 months to 9.6 months between 5-azacytidine and the three CCR subgroups.

[00227] The onset of the significant survival benefit occurred early in the present study with the Kaplan Meier curves for the 5-azacytidine and CCR groups separating permanently at approximately 3 months with nearly 80% of patients in the 5-azacytidine group having completed more than three cycles of treatment. Results obtained in the subgroup analyses for age, gender, FAB and WHO classification, karyotype; and LDH confirmed the robustness of the overall survival results achieved in the ITT population. The survival advantage in the 5-azacytidine group was maintained irrespective of IPSS cytogenetic risk group (favorable, intermediate, and poor), an important finding as abnormal karyotype is a frequent finding in MDS and a strong prognostic factor for a poorer outcome.

[00228] Findings in the secondary efficacy endpoints support the overall survival advantage demonstrated in the 5-azacytidine group. 5-Azacytidine treatment significantly prolonged the time to AML transformation or death and the time to transformation to AML compared with CCR. Significantly higher IWG-defined response rates were observed in the 5-azacytidine group compared with the CCR group, including complete or partial remission and major erythroid hematologic improvement. The superior response rates observed in the 5-azacytidine group were driven by notably lower rates in the LDara-C and BSC subgroups. Response rates in the small intensive chemotherapy subgroup were higher than those seen in the 5-azacytidine group. Remission and hematologic improvement rates also endured longer in the 5-azacytidine group than the CCR group.

[00229] RBC transfusion independence after baseline dependence was significantly higher in the 5-azacytidine group than in the CCR group, an important finding as transfusion dependency had been shown to be an significant marker of poorer outcome in MDS. No differences were observed between the 5-azacytidine and CCR group for platelet transfusion

independence, which was likely due to the small number of patients with baseline dependency. Additionally, although 5-azacytidine treatment was not associated with an increase in the proportion of patients with neutrophil improvement compared with the CCR group, a 33% reduction in the risk of infection requiring intravenous antimicrobials was observed in the 5-azacytidine group.

[00230] Grade 3 and 4 neutropenia was observed more frequently in the 5-azacytidine group than in the BSC subgroup, and at a similar rate compared with the LDara-C or intensive chemotherapy subgroups. Thrombocytopenia was also observed more commonly with 5-azacytidine than with BSC but less frequently than with LDara-C and intensive chemotherapy. However, despite the higher frequency of thrombocytopenia and neutropenia observed with 5-azacytidine compared with BSC, the overall occurrence of bleeding and infection was similar in both treatments.

[00231] Nonhematologic adverse events more commonly reported in the 5-azacytidine group than with the BSC subgroup, such as injection site reactions, nausea, and vomiting, were largely Grade 1-2 in severity, were well recognized events observed with 5-azacytidine treatment, and caused no patients to discontinue therapy. Generally, injection site reactions were easily managed by varying injection sites and by applying a post-injection cool or warm compress for 15 minutes.

[00232] The results demonstrated the first finding of an overall survival benefit in the treatment of MDS. Significantly longer overall survival was clearly shown with 5-azacytidine treatment compared with the CCR group, which comprised three other commonly used treatments in patients with higher risk MDS. The overall survival advantage was demonstrated irrespective of the CCR regimen (BSC, LDara-C, or intensive chemotherapy) and regardless of a good, intermediate, or poor IPSS cytogenetic risk. The results showing the overall survival benefit demonstrated with 5-azacytidine, given for a median of 9 cycles, was supported by a significant prolongation in time to AML transformation as well as increases in transfusion independence, complete and partial remissions, and major hematologic improvements. The significant increases in transfusion independence and hematologic improvement particularly suggested that decreasing cytopenias reduces the risk of their lethal complications, thus altering the natural disease course of MDS. These findings strongly established 5-azacytidine as the reference treatment in higher risk MDS, against which newer treatments will have to be compared or combined with in future trials in these patients.

[00233] **Table 2A. Baseline Demographics**

Parameter	Azacitidine N=179	Conventional Care Regimens			
		BSC Only N=105	LDAC, n=49	Intensive Chemo N=25	CCR Total N=179
Age (years)					
N	179	105	49	25	179
Median	69.0	70.0	71.0	65.0	70.0
Min, Max	42, 83	50, 88	56, 85	38, 76	38, 88
≤ 64, n (%)	57 (31.9)	24 (22.9)	7 (14.3)	12 (48.0)	43 (24)
≥ 65, n (%)	122 (68.1)	81 (77.1)	42 (85.7)	13 (52.0)	136 (76)
Gender – n (%)					
Male	132 (73.7)	67 (63.8)	35 (71.4)	17 (68.0)	119 (66.5)
Female	47 (26.3)	38 (36.2)	14 (28.6)	8 (32.0)	60 (33.5)
FAB Classification* – n (%)					
RAEB	104 (58.1)	68 (64.8)	25 (51.0)	10 (40.0)	103 (57.5)
RAEB-T	61 (34.1)	30 (28.6)	19 (38.8)	13 (52.0)	62 (34.6)
CMML	6 (3.3)	4 (3.8)	1 (2.0)	0	5 (2.8)
AML	1 (0.6)	0	0	1 (4.0)	1 (0.6)
IPSS – n (%)†					
Intermediate-1	5 (2.8)	9 (8.6)	2 (4.1)	2 (8.0)	13 (7.3)
Intermediate-2	76 (42.5)	46 (43.8)	21 (42.9)	3 (12.0)	70 (39.1)
High	82 (45.8)	46 (43.8)	21 (42.9)	18 (72.0)	85 (47.5)
Karyotype –n (%)					
Good	83 (46)	47 (45)	28 (57)	9 (36)	84 (47)
Intermediate	37 (21)	23 (22)	12 (25)	4 (16)	39 (22)
Poor	50 (28)	31 (29)	8 (16)	11 (44)	50 (28)
Missing	9 (5)	4 (4)	1 (2)	1 (4)	6 (3)
WHO Classification – n (%)					
RAEB-1	14 (7.8)	13 (12.4)	3 (6.1)	1 (4.0)	17 (9.5)
RAEB-2	98 (54.7)	60 (57.1)	24 (49.0)	11 (44.0)	95 (53.1)
CMMoL-1	1 (0.3)	0	0	0	0
CMMoL-2	10 (5.6)	3 (2.9)	0	2 (8.0)	5 (2.8)
AML	55 (30.7)	27 (25.7)	20 (40.8)	11 (44.0)	58 (32.4)
Indeterminate	1 (0.6)	2 (1.9)	2 (4.1)	0	4 (2.2)
ECOG Performance Status – n (%)					
0	78 (43.6)	36 (34.3)	29 (59.2)	15 (60.0)	80 (44.7)
1	86 (48.0)	59 (56.2)	17 (34.7)	10 (40.0)	86 (48.0)
2	13 (7.3)	8 (7.6)	2 (94.1)	0	10 (5.6)
Missing	2 (1.1)	2 (1.9)	1 (2.0)	0	3 (1.7)
Time Since Original Diagnosis (years) – n (%)					
< 1 year	92 (51.4)	53 (50.5)	28 (57.1)	14 (56.0)	95 (53.1)
1 to < 2 years	37 (20.7)	27 (25.7)	12 (24.5)	6 (24.0)	45 (25.1)
2 to < 3 years	20 (11.2)	6 (5.7)	3 (6.1)	1 (4.0)	10 (5.6)
≥ 3 years	30 (16.8)	19 (18.1)	6 (12.2)	4 (16.0)	29 (16.2)

* Another 3.9% and 4.5% of patients in the azacitidine and CCR groups, respectively, had myeloproliferative disease or were disease was indeterminate.

† Another 8.9% and 6.2% of patients in the azacitidine and CCR groups, respectively, had disease not applicable to IPSS or were indeterminate

[00234] Table 2B: Baseline Demographics by Investigator Pre-Selection

Investigator Pre-Selection	BSC Only (n=222)		LDara-C (n=94)		Intensive Chemotherapy (IC) (n=42)	
Randomization	Azacitidine N=117	BSC N=105	Azacitidine N=45	LDara-C N=49	Azacitidine N=17	IC N=25
Parameter						
Age (years) N	117	105	45	49	17	25
Median	69.0	70.0	69.0	71.0	63.0	65.0
Min, Max	52, 83	50, 88	42, 92	56, 85	45, 79	38, 76
≤ 64, n (%)	33 (28.2)	24 (22.9)	14 (31.1)	7 (14.3)	10 (58.8)	12 (48.0)
≥ 65, n (%)	84 (71.8)	81 (77.1)	31 (68.9)	42 (85.7)	7 (41.2)	13 (52.0)
Gender – n (%) Male	81 (69.2)	67 (63.8)	39 (86.7)	35 (71.4)	12 (70.6)	17 (68.0)
Female	36 (30.8)	38 (36.2)	6 (13.3)	14 (29.6)	5 (29.4)	9 (32.0)
FAB Classification – n (%) RAEB	69 (59.0)	68 (64.8)	27 (60.0)	25 (51.0)	8 (47.1)	10 (40.0)
RAEB-T	38 (32.5)	30 (28.6)	15 (33.3)	19 (38.8)	8 (47.1)	13 (52.0)
CMML	5 (4.3)	4 (3.8)	1 (2.2)	1 (2.0)	0 (0.0)	0 (0.0)
AML	0 (0.0)	0 (0.0)	1 (2.2)	0 (0.0)	0 (0.0)	1 (4.0)
IPSS – n (%) Intermediate-1	4 (3.4)	9 (8.6)	1 (2.2)	2 (4.1)	0 (0.0)	2 (8.0)
Intermediate-2	48 (41.0)	46 (43.8)	22 (48.9)	21 (42.9)	6 (35.3)	3 (12.0)
High	57 (48.7)	46 (43.8)	19 (42.2)	21 (42.9)	6 (35.3)	18 (72.0)
Karyotype – n (%) Good	53 (45.3)	47 (44.8)	24 (53.3)	28 (57.1)	6 (35.3)	9 (36.0)
Intermediate	25 (21.4)	23 (21.9)	7 (15.6)	12 (24.5)	5 (29.4)	4 (16.0)
Poor	33 (28.2)	31 (29.5)	13 (28.9)	8 (16.3)	4 (23.5)	11 (44.0)
Missing	6 (5.1)	4 (3.8)	1 (2.2)	1 (2.0)	2 (11.8)	1 (4.0)

Investigator Pre-Selection	BSC Only (n=222)		LDara-C (n=94)		Intensive Chemotherapy (IC) (n=42)	
Randomization	Azacitidine N=117	BSC N=105	Azacitidine N=45	LDara-C N=49	Azacitidine N=17	IC N=25
WHO Classification – n (%) RAEB-1	8 (6.8)	13 (12.4)	3 (6.7)	3 (6.1)	3 (17.6)	1 (4.0)
RAEB-2	63 (53.8)	60 (57.1)	27 (60.0)	24 (49.0)	8 (47.1)	11 (44.0)
CMML-1	1 (0.9)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
CMML-2	8 (6.8)	3 (2.9)	1 (2.2)	0 (0.0)	1 (5.9)	2 (8.0)
AML	36 (30.8)	27 (25.7)	14 (31.1)	20 (40.8)	5 (29.4)	11 (44.0)
Indeterminate	1 (0.9)	2 (1.9)	0 (0.0)	2 (4.1)	0 (0.0)	0 (0.0)
ECOG Performance Status – n (%) 0	47 (40.2)	36 (34.3)	21 (46.7)	29 (59.2)	10 (58.8)	15 (60.0)
1	59 (50.4)	59 (56.2)	21 (46.7)	17 (34.7)	6 (35.3)	10 (40.0)
2	11 (9.4)	8 (7.6)	1 (2.2)	2 (4.1)	1 (5.9)	0 (0.0)
Missing	0 (0.0)	2 (1.9)	2 (4.4)	1 (2.0)	0 (0.0)	0 (0.0)
Time Since Original Diagnosis (years) – n (%) < 1 year	53 (45.3)	53 (50.5)	29 (64.4)	28 (57.1)	10 (58.8)	14 (56.0)
1 to < 2 years	29 (24.8)	27 (25.7)	7 (15.6)	12 (24.5)	1 (5.9)	6 (24.0)
2 - < 3 years	14 (12.0)	6 (5.7)	4 (8.9)	3 (6.1)	2 (11.8)	1 (4.0)
≥ 3 years	21 (17.9)	19 (18.1)	5 (11.1)	6 (12.2)	4 (23.5)	4 (16.0)

6.2 Example 2

[00235] This study evaluates gene methylation biomarkers and prolonged survival in patients with certain MDS (*e.g.*, higher risk MDS) treated with 5-azacytidine. Hypomethylation is believed to be a molecular mechanism of action of 5-azacytidine; accordingly, research on the effect of methylation status particular genes, and of gene combinations, is conducted. Both DNA methylation and RNA methylation are contemplated as potential biomarkers.

[00236] In one embodiment, a study is performed to examine whether baseline DNA and/or RNA methylation levels influence overall survival (OS) as well as the interaction between gene promotor methylation levels and treatment (*e.g.*, 5-azacytidine or CCR). For example, methylation is determined for 5 genes: *CDKN2B (p15)*, *SOCS1*, *CDH1 (E-cadherin)*, *TP73*, and *CTNNA1 (alpha-catenin*), in pre-treatment bone marrow aspirates of patients enrolled in a clinical study using quantitative real-time methylation specific PCR (qMSP). The influence of methylation on OS is assessed using Cox proportional hazards models and Kaplan-Meier (KM) methodology.

[00237] The number of patients (for 5-azacytidine and CCR) having nucleic acid sufficient for analysis of these genes is determined. For example, methylation is detected in a specific percentage of patients for *CDKN2B, SOCS1*, *CDH1*, *TP73*, and *CTNNA1*. Differences in methylation levels between the treatment arms are determined. The OS benefit for 5-azacytidine treatment is determined for patients who are positive and negative for methylation at these genes. It is determined whether the presence of methylation is associated with improvement in OS in the CCR group (prognostic indicator of good outcome). The existence and magnitude of any effect is compared to the 5-azacytidine group, which may suggest an interaction between DNA and/or RNA methylation and treatment.

[00238] OS improvement is assessed with 5-azacytidine treatment in patients with methylation at any of these genes, and HR of death for methylation is determined. The frequency of methylation of particular genes allows for examination of the influence of methylation level on OS and treatment effect. For example, for particular genes, lower levels of methylation may be associated with the longest OS and the greatest OS benefit from 5-azacytidine treatment, compared with the absence of methylation. Influence of methylation level on OS may be assessed in each IPSS cytogenetic subgroup (good, intermediate, and poor). For example, the influence of methylation on OS may be strongest in the "poor" risk group, where risk of death is greatest.

[00239] Such data and analysis may indicate, *e.g.*, that patients with lower levels of methylation may derive greater benefit from 5-azacytidine. Molecular biomarkers may be important in MDS, *e.g.*, as indicators of disease prognosis and predictors of response to epigenetic therapy.

6.3 Example 3

[00240] Studies were done to determine if baseline DNA methylation influenced overall survival in MDS subjects from the AZA 001 study (Example 1). Potential correlations between gene promoter methylation levels and treatment efficacy (AZA or conventional care regimens (CCR)) were evaluated. Specifically, the DNA methylation of 5 genes, *CDKN2B (p15)*, *SOCS1*, *CDH1 (E-cadherin)*, *TP73*, and *CTNNA1 (α-catenin)*, in pre-treatment baseline bone marrow aspirates of 303 patients were evaluated, using quantitative real-time methylation specific PCR. The influence of methylation levels on overall survival was assessed using Cox proportional hazards models and Kaplan-Meier methodology.

[00241] In one embodiment, the DNA methylation of baseline bone marrows from patients were measured using the Illumina Infinium Methylation27 DNA methylation arrays (e.g., Beadarray). In one embodiment, other known technologies for measuring DNA methylation are used in a method provided herein.

[00242] Two hundred and seventy-one patients (AZA [n=136] and CCR [n=135]) had sufficient DNA for analysis of all 5 genes. DNA methylation was detected in 82% of patients' samples for *CDKN2B*, 54% for *SOCS1*, 87% for *CDH1*, 2% for *TP73*, and 10% for *CTNNA1*. Overall, methylation levels did not differ between the two treatment arms. There was an OS benefit for AZA vs. CCR treatment in patients regardless of methylation status at these five genes. The presence of abnormal methylation at any locus was associated with a slightly improved OS in both the AZA and CCR groups (prognostic indicator of good outcome); however, this effect was more pronounced in the AZA group. This suggests an interaction between DNA methylation and treatment. OS improved to a greater extent with AZA treatment in patients with methylation of any one of 4 of these 5 genes (*SOCS1*, *CDH1*, *TP73*, and *CTNNA1*), with HR of death for methylation 0.54–0.93 (HR for *CDKN2B* is 1.01). Two genes were infrequently methylated (*TP73*, *CTNNA1*), but the frequency of methylation of *CDKN2B*, *SOCS1*, and *CDH1* allowed examination of the influence of methylation level on OS and treatment effect. For these three genes, the presence of methylation was associated with significant improvement in OS with AZA treatment compared with no methylation. Lower levels of methylation (Table 3) were associated with the greatest OS

benefit from AZA; this benefit diminished as methylation levels increased (data for *CDH1* are shown in Table 3; this pattern was similar for the other genes). This influence of methylation level on OS was seen in all IPSS cytogenetic subgroups, but was strongest in the poor risk group where risk of death was greatest.

[00243] The overall survival benefit observed with AZA versus CCR appeared to be independent of pre-treatment baseline methylation status of the five genes analyzed. However, increased methylation in pre-treatment baseline bone marrow was associated with worse overall survival, and patients with lower levels of methylation treated with AZA had the best overall survival, suggesting that these patients may derive greater benefit from AZA.

[00244] In one embodiment, the relationship between *CDH1* gene methylation and prolonged overall survival was analyzed and summarized in Table 3. The hazard ratios and 95% Cl of the analysis of *CDH1* gene methylation and prolonged overall survival and AML transformation are summarized in Figure 4.

[00245] Additional studies were done, for example, with *CDKN2B* promoter DNA methylation density in pre-treatment baseline bone marrows from patients in the AZA 001 study, looking at association of overall survival and response with AZA or CCR treatments. Specifically, the percentage of DNA methylation was determined for 18 CpG units in the *CDKN2B* promoter of genomic DNA isolated from 295 pre-treatment bone marrow aspirates of consenting patients using the Sequenom EpiTYPER® platform. A weighted average of methylation levels in these 18 CpG units was used to evaluate association with clinical outcome, with the number of individual CpG dinucleotides comprising each CpG unit used as the weight for that unit. Kaplan-Meier methods were used for survival estimates. Cox proportional hazards models, stratified by the randomization factors FAB subtype and international prognostic scoring system (IPSS) group, were used to estimate hazard ratios (HRs). It appeared that the overall survival and hematologic response benefits observed with 5-azacytidine vs. CCR were independent of baseline methylation status of *CDKN2B*, and that a similar proportion of patients experienced increased hematologic response rates with 5-azacytidine in each methylation group.

[00246] In sum, it appeared that the survival benefit of 5-azacytidine treatment of MDS was observed in all methylation levels, but was greatest for patients with lower levels of DNA methylation pre-treatment. This correlation with methylation level was also seen for time to AML progression, which often showed a stronger correlation than the correlation for overall survival. It appeared that the OS benefit observed with AZA vs. CCR was independent of methylation status of the five genes analyzed. Increasing methylation was

associated with worse OS. Patients with lower levels of methylation treated with AZA had the best OS and may derive greater benefit from AZA. These results underscore the complexity of using molecular biomarkers to predict response to epigenetic therapy.

Table 3: CDH1 Gene Methylation and Prolonged OS

CDH1	Deaths n/N	KM Median Survival (mos)	Hazard Ratio (95% CI)	P Value
Population Analyzed (N=271)	147/271	17.2		
Any Methylation				
*No (0)	26/36	10.9	1.00	
Yes (>0)	121/235	18.4	0.54 (0.35, 0.85)	0.008
Methylation Level				
*0	26/36	10.9	1.00	
>0 and ≤ 0.62	33/77	25.1	0.44 (0.26, 0.77)	0.004
>0.62 and ≤ 1.62	39/79	18.7	0.48 (0.29, 0.81)	0.006
>1.62	49/79	13.2	0.71 (0.43, 1.19)	0.193
Treatment/Methylation Interaction				
*CCR: 0	16/20	7.2	1.00	
AZA: 0	10/16	12.0	0.68 (0.30, 1.55)	0.353
CCR: >0 and ≤0.62	17/35	19.5	0.51 (0.25, 1.06)	0.071
AZA: >0 and ≤0.62	16/42	26.3	0.26 (0.12, 0.53)	<0.001
CCR: >0.62 and ≤1.62	19/35	18.7	0.40 (0.20, 0.83)	0.013
AZA: >0.62 and ≤1.62	20/44	25.1	0.39 (0.20, 0.76)	0.006
CCR: >1.62	30/45	12.3	0.74 (0.38, 1.41)	0.355
AZA: >1.62	19/34	17.1	0.44 (0.22, 0.89)	0.022

* Reference group.

6.4 Example 4

[00247] In this study, analysis of DNA methylation patterns in the baseline bone marrow (BM) of MDS patients from the AZA-001 trial was expanded to a greater group of genes to identify a DNA methylation-based predictive signature or pattern, predictive of overall survival (OS) or other clinical benefits in AZA-treated patients.

[00248] Air-dried drops of bone marrow aspirate were collected on unstained slides at baseline (pre-treatment), and at every 16 weeks post-therapy (Figure 5). DNA methylation levels of 27,578 genomic loci were measured in pre-treatment unpurified bone marrow

aspirates of 129 patients (AZA [n=59] and CCR [n=70]) using Illumina Infinium Methylation27 Beadarray. The data were randomly divided into training data set (n=95: AZA [n=38] and CCR [n=57]) and validation data set (n=34: AZA [n=21] and CCR [n=13]). A DNA methylation signature predictive of OS in AZA-treated patients was identified using uniCox algorithm (Tibshirani, Stat Appl Genet Mol Biol. 2009) first in the training data and then re-evaluated in the validation data (Figure 5). The influence of the signature on OS was assessed using Cox proportional hazards models. All statistical analyses were carried out in R (R Foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org).

[00249] To identify baseline DNA methylation signature that correlates with overall survival in the 38 patients treated with 5-azacytidine, 'uniCox' R package was used. *See, e.g.*, Tibshirani, "Univariate Shrinkage in the Cox Model for High Dimensional Data," *Statistical Applications in Genetics and Molecular Biology*, 8(1), 2009, for a description of an algorithm. The identified signature contained a total of 214 loci mapped to 187 genes. A heatmap of the signature in the baseline samples is shown in Figure 6. The coefficients for each locus within the signature and their mean methylation ratios in the training data are listed in Table 4 (the first column of the table lists ID numbers from Illumina BeadArray and each ID represents one CpG site which is subject to DNA methyaltion). A predicted score for a new sample was calculated as follows:

$$score = \sum_{i=1}^{p} coef_i \times (\beta_i^* - \bar{\beta}_i)$$

where β_i^* was the methylation ratio for the i^{th} locus in the new sample and $\overline{\beta}_i$ was the mean methylation ratio for the i^{th} locus in the training data. In the validation data set, this predicted score was used to assess its correlation with overall survival, *e.g.*, in a Cox regression model.

Table 4. Coefficients for gene signature predictive of overall survival.

	Gene	coef	center
cg18328933	ABHD14A	7.35000	0.534
cg11879188	ABO	0.31700	0.576
cg20807545	ADAMTS18	5.56000	0.390
cg21542793	ADRA2B	5.95000	0.417
cg01386493	ADRB3	3.59000	0.505
cg17356252	AIRE	0.05320	0.613
cg12061236	AKAP12	0.50900	0.241
cg15799267	ALOX15B	2.43000	0.342
cg11052143	ALS2CR11	4.27000	0.717
cg15780361	ALS2CR11	4.69000	0.651
cg20191453	AMT	3.20000	0.410
cg19948393	ANKRD33	3.09000	0.717
cg18133957	APC2	−1.47000	0.555
cg16536918	AVP	2.95000	0.561
cg10660256	BHMT	0.36100	0.495
cg04727522	C18orf22	−0.25200	0.439
cg21300318	C19orf30	0.88600	0.431
cg21921474	C1orf172	5.88000	0.504
cg09851465	C1orf87	3.69000	0.333
cg26143719	C1QTNF6	2.07000	0.645
cg17940740	C22orf27	1.50000	0.425
cg01353448	C7orf16	−0.06580	0.677
cg08972170	C7orf41	5.80000	0.321
cg23124451	CBX7	2.58000	0.449
cg02849695	CCDC19	2.25000	0.680
cg16404106	CCDC81	2.16000	0.433
cg05436231	CD164L2	3.23000	0.468
cg11667754	CDH1	0.04690	0.192
cg06421800	CDKN2B	8.04000	0.400
cg06958829	CHAD	3.49000	0.447
cg27382389	CHRNG	1.47000	0.429
cg15364618	CIDEB	5.03000	0.207
cg03679734	CKMT1B	−3.29000	0.330
cg10978355	CKMT2	2.32000	0.378
cg05228408	CLCN6	2.09000	0.327
cg07384961	CLDN6	3.12000	0.257
cg10282491	CLDN9	2.74000	0.629
cg11563860	CNTN4	−9.65000	0.290
cg00983520	CPT1B	0.86500	0.424
cg16545105	CRHBP	3.73000	0.285
cg04559909	CXCL5	0.51200	0.525
cg10088985	CXCL5	0.88400	0.173
cg20322977	CYP26C1	0.43200	0.538
cg18182399	DES	0.38900	0.348
cg10303487	DPYS	2.23000	0.278
cg18396533	DYDC1	0.55600	0.320
cg08529852	EGFL7	3.29000	0.415

	Gene	coef	center
cg19514469	ELMO3	0.10800	0.737
cg25483003	ENTPD2	0.48500	0.241
cg17200465	ENTPD3	1.15000	0.480
cg15626350	ESR1	0.49800	0.684
cg01805282	EYA4	2.29000	0.181
cg00415993	F2RL2	1.85000	0.424
cg13271951	FAM57B	0.86900	0.504
cg04337944	FBLN1	1.36000	0.459
cg01420388	FBXO2	0.75900	0.635
cg23065097	FKBP1B	0.82100	0.481
cg21306775	FLJ44881	−0.21000	0.547
cg04001333	FLVCR2	7.76000	0.595
cg23771929	FREQ	0.58700	0.361
cg20692569	FZD9	2.18000	0.523
cg01601573	GAB1	0.02620	0.410
cg10016608	GAS2L2	4.81000	0.328
cg13434842	GATA4	3.47000	0.480
cg01169778	GBGT1	−2.86000	0.495
cg07378350	GDF5	4.38000	0.502
cg22459146	GHSR	2.93000	0.354
cg00943909	GNAS	−0.42900	0.566
cg10056627	GNMT	2.33000	0.169
cg10862848	GNMT	0.14800	0.544
cg14338887	GNMT	3.19000	0.159
cg24101359	GNMT	2.87000	0.149
cg27588902	GNMT	0.95900	0.237
cg20395072	GNPNAT1	1.87000	0.355
cg11340260	GP1BA	1.93000	0.418
cg21870884	GPR25	1.79000	0.545
cg14859460	GRM6	4.04000	0.251
cg15674997	GRM6	1.52000	0.536
cg04987894	GSTM5	0.78600	0.374
cg02860342	HCN4	2.89000	0.391
cg10146929	HIST1H1A	4.73000	0.294
cg00767581	HOXD4	3.45000	0.375
cg12127282	HOXD4	2.33000	0.257
cg24642523	HSPA2	0.30100	0.394
cg18786940	HTATIP2	1.46000	0.399
cg06291867	HTR7	2.07000	0.237
cg20977864	HYDIN	0.71400	0.550
cg16003238	IGDCC3	2.10000	0.615
cg08463485	ILDR1	1.23000	0.492
cg10074409	IRF6	2.75000	0.271
cg15302379	KAZALD1	4.18000	0.577
cg26162582	KCNA6	0.03310	0.228
cg01643580	KCNK3	−1.27000	0.325
cg17820828	KCNQ1	3.06000	0.618
cg19728223	KCNQ1	0.80100	0.207
cg18530324	KIAA0427	2.11000	0.622
cg03602500	KIR3DX1	−3.53000	0.637
cg18901980	KRT25	−9.17000	0.695
cg07007400	KRT7	1.48000	0.201
cg20050826	KRT72	0.25100	0.511
cg25947945	LAD1	4.37000	0.271
cg14289461	LAMA4	0.23500	0.450
cg23696949	LAMC2	1.32000	0.468
cg16639185	LGTN	4.81000	0.539
cg20442697	LRRC17	0.00177	0.511
cg15261665	LTF	0.36400	0.227
cg17527798	LTF	1.88000	0.254
cg13727946	MBD3L1	−0.72600	0.360
cg26465611	MEGF10	0.64700	0.445
cg05949660	MICAL1	2.46000	0.567
cg12437481	MRPL28	−0.38900	0.508
cg24378421	MTMR9	2.57000	0.477
cg15842276	MTNR1B	0.45200	0.586
cg15202954	NALCN	−1.50000	0.267
cg06952310	NCAN	5.60000	0.521
cg22580512	NCOR2	6.48000	0.489
cg18081258	NDRG2	1.13000	0.366
cg07109801	NDUFAF3	−5.80000	0.400
cg17830308	NEUROG1	3.24000	0.175
cg15501381	NGB	1.56000	0.640
cg20656113	NPFFR2	−0.10200	0.262
cg26401870	NPM2	0.75700	0.285
cg14506552	NPPB	6.10000	0.541
cg12876594	NPR2	1.58000	0.661

	Gene	coef	center
cg24194775	NPR2	0.80800	0.655
cg12770741	NXN	1.47000	0.588
cg08409225	OBFC2B	3.50000	0.308
cg21387281	OCFB	1.83000	0.398
cg02250594	ONECUT2	0.35900	0.285
cg14882700	OTOP1	6.14000	0.400
cg20267561	OXT	2.39000	0.514
cg26955850	OXT	0.44400	0.255
cg23239444	PACSIN1	3.39000	0.332
cg18361093	PAOX	2.82000	0.233
cg12554573	PARP3	4.79000	0.492
cg24401441	PAX1	2.02000	0.512
cg20366906	PCDH8	8.21000	0.346
cg24076884	PCDHAC2	−0.43900	0.323
cg17861230	PDE4C	0.62500	0.522
cg20615832	PF4V1	0.09780	0.435
cg11377136	PKDREJ	2.02000	0.422
cg14159672	PM20D1	1.87000	0.390
cg14893161	PM20D1	0.54000	0.484
cg08030682	POMC	0.52300	0.507
cg16302441	POMC	1.44000	0.390
cg17791651	POU3F1	0.14200	0.508
cg18636641	PPAPDC3	6.17000	0.646
cg23352030	PRIC285	−2.57000	0.674
cg09309269	PSMD11	−5.35000	0.226
cg07612655	PTGIS	2.36000	0.382
cg15379633	RAB36	1.82000	0.337
cg22396755	RAP1GAP	0.35000	0.181
cg16154416	RASGEF1	7.38000	0.291
cg23082877	RASIF1	1.41000	0.327
cg21835643	RBPJL	3.58000	0.261
cg00055233	RLN1	0.65000	0.280
cg17006282	RPL36	−3.47000	0.646
cg04431133	RPL36AL	−1.52000	0.512
cg26417554	RPUSD3	−2.74000	0.613
cg15787039	SCG5	2.69000	0.588
cg03387723	SCMH1	7.02000	0.423
cg21604042	SCUBE3	0.37300	0.307
cg24019851	SEMA3B	4.90000	0.634
cg11300809	SGPP2	4.47000	0.442
cg16609957	SHROOM1	4.13000	0.336
cg05106502	SKAP1	1.35000	0.420
cg14391622	SLC12A8	3.37000	0.446
cg10141715	SLC5A8	1.04000	0.366
cg09816471	SNN	2.26000	0.272
cg27380758	SORBS3	1.78000	0.362
cg15206445	SPG7	−8.43000	0.441
cg20531804	SPINT1	5.08000	0.455
cg15403517	SRD5A2	1.89000	0.423
cg08558340	SRRT	2.93000	0.517
cg17586860	SSTR4	2.61000	0.499
cg04471507	STMN1	0.63500	0.318
cg25608041	TBC1D1	0.06040	0.587
cg19242268	TCEA2	3.01000	0.472
cg06143901	TCF15	2.18000	0.476
cg20372517	TFAP2E	1.69000	0.407
cg00386408	TGFBI	0.34000	0.356
cg15853125	TIAM1	0.61600	0.185
cg04355435	TMEM125	3.57000	0.225
cg05322222	TMEM151A	−1.50000	0.218
cg06194808	TMEM184A	1.86000	0.690
cg02828104	TMEM189	3.51000	0.368
cg19686152	TMOD3	−3.52000	0.482
cg19504245	TNNT1	0.90700	0.178
cg18059933	TP53INP1	1.17000	0.221
cg21081971	TRPC4	−0.15500	0.593
cg10832407	TRPM3	0.04450	0.385
cg14419187	UNC80	−2.05000	0.325
cg11108890	VAMP5	5.11600	0.268
cg25651505	VAMP5	4.61000	0.283
cg16869108	VHL	−3.72000	0.686
cg01718139	VSTM1	−0.32700	0.472
cg02237119	WBSCR27	1.39000	0.584
cg07054641	WDR52	4.17600	0.593
cg15044041	WDR52	1.83000	0.638
cg01693350	WT1	1.87000	0.605
cg04096767	WT1	4.25600	0.523

	Gene	coef	center
cg04456238	WT1	2.46000	0.586
cg05222924	WT1	1.19000	0.399
cg06516124	WT1	0.18500	0.305
cg12006284	WT1	3.12000	0.509
cg13301003	WT1	4.70000	0.469
cg15446391	WT1	4.17000	0.528
cg16463460	WT1	0.38900	0.486
cg22533573	WT1	1.26000	0.353
cg25094569	WT1	0.03940	0.222
cg25782229	WT1	1.85000	0.471
cg24641352	ZFP41	1.80000	0.374
cg13928306	ZNF205	3.33000	0.681
cg01185080	ZNF710	4.46000	0.435

[00250] A baseline DNA methylation signature predictive of overall survival in AZA-treated patients was identified based on a training set of 38 AZA-treated patients and re-evaluated in a test set of 21 AZA-treated patients. The signature contains 214 genomic loci representing 187 genes, including, *ABHD14A*, *ABO*, *ADAMTS18*, *ADRA2B*, *ADRB3*, *AIRE*, *AKAP12*, *ALOX15B*, *ALS2CR11*, *AMT*, *ANKRD33*, *APC2*, *AVP*, *BHMT*, *C18orf22*, *C19orf30*, *C1orf172*, *C1orf87*, *C1QTNF6*, *C22orf27*, *C7orf16*, *C7orf41*, *CBX7*, *CCDC19*, *CCDC81*, *CD164L2*, *CDH1*, *CDKN2B*, *CHAD*, *CHRNG*, *CIDEB*, *CKMT1B*, *CKMT2*, *CLCN6*, *CLDN6*, *CLDN9*, *CNTN4*, *CPT1B*, *CRHBP*, *CXCL5*, *CYP26C1*, *DES*, *DPYS*, *DYDC1*, *EGFL7*, *ELMO3*, *ENTPD2*, *ENTPD3*, *ESR1*, *EYA4*, *F2RL2*, *FAM57B*, *FBLN1*, *FBXO2*, *FKBP1B*, *FLJ44881*, *FLVCR2*, *FREQ*, *FZD9*, *GAB1*, *GAS2L2*, *GATA4*, *GBGT1*, *GDF5*, *GHSR*, *GNAS*, *GNMT*, *GNPNAT1*, *GP1BA*, *GPR25*, *GRM6*, *GSTM5*, *HCN4*, *HIST1H1A*, *HOXD4*, *HSPA2*, *HTATIP2*, *HTR7*, *HYDIN*, *IGDCC3*, *ILDR1*, *IRF6*, *KAZALD1*, *KCNA6*, *KCNK3*, *KCNQ1*, *KIAA0427*, *KIR3DX1*, *KRT25*, *KRT7*, *KRT72*, *LAD1*, *LAMA4*, *LAMC2*, *LGTN*, *LRRC17*, *LTF*, *MBD3L1*, *MEGF10*, *MICAL1*, *MRPL28*, *MTMR9*, *MTNR1B*, *NALCN*, *NCAN*, *NCOR2*, *NDRG2*, *NDUFAF3*, *NEUROG1*, *NGB*, *NPFFR2*, *NPM2*, *NPPB*, *NPR2*, *NXN*, *OBFC2B*, *OGFR*, *ONECUT2*, *OTOP1*, *OXT*, *PACSIN1*, *PAOX*, *PARP3*, *PAX1*, *PCDH8*, *PCDHAC2*, *PDE4C*, *PF4V1*, *PKDREJ*, *PM20D1*, *POMC*, *POU3F1*, *PPAPDC3*, *PRIC285*, *PSMD11*, *PTGIS*, *RAB36*, *RAP1GAP*, *RASGRF1*, *RASIP1*, *RBPJL*, *RLN1*, *RPL36*, *RPL36AL*, *RPUSD3*, *SCG5*, *SCMH1*, *SCUBE3*, *SEMA3B*, *SGPP2*, *SHROOM1*, *SKAP1*, *SLC12A8*, *SLC5A8*, *SNN*, *SORBS3*, *SPG7*, *SPINT1*, *SRD5A2*, *SRRT*, *SSTR4*, *STMN1*, *TBC1D1*, *TCEA2*, *TCF15*, *TFAP2E*, *TGFBI*, *TIAM1*, *TMEM125*, *TMEM151A*, *TMEM184A*, *TMEM189*, *TMOD3*, *TNNT1*, *TP53INP1*, *TRPC4*, *TRPM3*, *UNC80*, *VAMP5*, *VHL*, *VSTM1*, *WBSCR27*, *WDR52*, *WT1*, *ZFP41*, *ZNF205*, and *ZNF710*. A list of enriched bio-groups for predicting overall survival is summarized in Table 5.

Table 5. Enriched Bio-Groups Predictive of Overall Survival

BioGroup Name	Source	Common Genes	p-value
Cadherin-like	InterPro	15	1.50E-25
homophilic cell adhesion	GO	15	1.50E-24
chr5q31	Broad MSigDB - Positional Gene Sets	16	9.50E-24
cell-cell adhesion	GO	16	1.50E-20
calcium ion binding	GO	15	1.10E-14
muscle contraction	GO	15	1.70E-11
E12 binding site geneset 2	Broad MSigDB - Regulatory Motifs	45	7.00E-10
blood circulation	GO	14	1.80E-09
RSRFC4 binding site geneset 3	Broad MSigDB - Regulatory Motifs	15	2.60E-08
cell differentiation	GO	35	4.00E-08
Leukotriene B4 receptor	InterPro	2	5.40E-07
cell-cell signaling	GO	19	6.80E-07
regulation of muscle contraction	GO	7	8.10E-07
NRSF binding site geneset 1	Broad MSigDB - Regulatory Motifs	7	1.20E-06
E47 binding site geneset 2	Broad MSigDB - Regulatory Motifs	6	3.60E-06
regulation of blood pressure	GO	7	5.00E-06
Troponin	InterPro	3	5.20E-06
LMO2COM binding site geneset 1	Broad MSigDB - Regulatory Motifs	6	6.90E-06
epithelial cell differentiation	GO	9	8.40E-06
Neuroactive Ligand Receptor Interaction	Broad MSigDB - Canonical Pathways	10	8.40E-06

[00251] A DNA methylation score was calculated for each patient as the weighted average of the DNA methylation levels of these 214 genomic loci, with weights optimized in the training data set. In the training set, the DNA methylation score was significantly (hazard

ratio [HR]=2.34 [95% confidence interval: 1.54, 3.54]; p = 5.8e-5) associated with overall survival (OS) and remained significant (HR=3.25 [1.65, 6.38]; p = 0.00062) in a multivariate analysis, adjusting for the clinical co-variates: sex, cytogenetic score, ECOG grade, and baseline RBC transfusion status. The DNA methylation score was not predictive of OS in patients treated with conventional care (p = 0.81), demonstrating the specificity of this marker to 5-azacytidine therapy. When applied to the validation set, the association of the DNA methylation score with OS remained marginally significant (HR=1.66 [0.99, 2.79]; p = 0.053) in a univariate analysis, but became less significant (p = 0.52) in a multivariate analysis. For the majority of the loci in the signature, lower DNA methylation correlated with better survival outcome. A predictive signature was also generated using the entire dataset (training and test sets combined), resulting in 53 loci, representing 39 genes, including *WT1* (Figure 7).

[00252] DNA methylation of *WT1* loci alone was predictive of OS in both univariate (HR=1.47 [1.00, 2.16]; p = 0.048) and multivariate (HR=2.04 [1.14, 3.64]; p = 0.016) analyses of AZA-treated patients in the training data set. DNA methylation of *WT1* loci remained as a significant (HR=2.87 [1.24, 6.64]; p = 0.014) predictor of OS in the test data set in univariate analysis, but became less significant (p = 0.35) in a multivariate analysis. As with the signature containing 214 loci, DNA methylation of *WT1* loci was not predictive of OS in patients treated with conventional care (p = 0.64). All performance results are summarized in Table 6.

[00253] In the combined dataset, the DNA methylation signature was significantly (p = 0.000278) associated with OS in a multivariate analysis, including addition of clinical co-variates. Methylation of *WT1* loci had predictive value in univariate and multivariate analyses.

[00254] In sum, specific DNA methylation patterns were observed in certain higher-risk MDS patients, which were predictive of overall survival or other clinical benefits in patients upon 5-azacytidine treatment. For example, a DNA methylation signature was identified in the unpurified pre-treatment bone marrow aspirate samples, which was shown to be predictive of OS of 5-azacytidine-treated higher-risk MDS patients.

Table 6. Performance of DNA methylation signatures on predicting outcome

		Methylation Signature (n=214)			WT1 loci only (n=12)	
		p	HR (95% CI)[3]		p	HR (95% CI)[3]
AZA arm (n=59)						
Training Data (n=38)	p^1 =	5.8E-05	2.34 [1.54, 3.54]	p^1 =	0.048	1.47 [1, 2.16]
	p^2 =	0.00062	3.25 [1.65, 6.38]	p^2 =	0.016	2.04 [1.14, 3.64]
Validation Data (n=21)	p^1 =	0.053	1.66 [0.99, 2.79]	p^1 =	0.014	2.87 [1.24, 6.64]
	p^2 =	0.52	1.55 [0.4, 5.91]	p^2 =	0.35	1.82 [0.51, 6.41]
CCR arm (n=70)						
Training Data (n=57)	p^1 =	0.32	1.26 [0.8, 1.98]	p^1 =	0.99	1 [0.69, 1.46]
	p^2 =	0.25	0.7 [0.38, 1.28]	p^2 =	0.26	0.78 [0.5, 1.21]
Validation Data (n=13)	p^1 =	0.27	0.6 [0.25, 1.47]	p^1 =	0.21	0.49 [0.16, 1.49]
	p^2 =	0.16	0.11 [0.01, 2.44]	p^2 =	0.26	NA[4]
All patients (n=129)						
AZA arm (n=59)	p^1 =	3.9E-06	2.09 [1.53, 2.86]	p^1 =	0.0024	1.72 [1.21, 2.45]
	p^2 =	0.00019	2.29 [1.48, 3.55]	p^2 =	0.0074	1.92 [1.19, 3.09]
CCR arm (n=70)	p^1 =	0.81	1.05 [0.71, 1.54]	p^1 =	0.64	0.92 [0.66, 1.29]
	p^2 =	0.65	0.9 [0.56, 1.44]	p^2 =	0.59	0.91 [0.65, 1.28]

[1] p values from univariate Cox regression models.
[2] p values from Cox regression models adjusting for other clinical co-variates.
[3] The HRs and their 95% CIs are calculated using the standardized methylation scores.
[4] The model did not converge

[00255] While the examples have been particularly shown and described with reference to a number of embodiments, it would be understood by those skilled in the art that changes in the form and details may be made to the various embodiments disclosed herein and that the various embodiments disclosed herein are not intended to act as limitations on the scope of the claims. All patents, publications, and other references cited herein are incorporated by reference herein in their entireties.

WHAT IS CLAIMED IS:

1. A method of treating a subject having a disease, comprising:

(i) obtaining cells from the subject;

(ii) measuring the level of methylation of one or more gene(s) in the cells;

(iii) comparing the methylation level of one or more gene(s) with a reference methylation value or a methylation pattern and selecting a therapeutic agent to be administered to the subject; and

(iv) administering the therapeutic agent to the subject in need thereof.

2. The method of claim 1, wherein the disease is a myelodysplastic syndrome.

3. The method of claim 1, wherein the disease is higher-risk myelodysplastic syndrome.

4. The method of claim 1, wherein the disease is cancer.

5. The method of claim 1, wherein the disease is a blood-born tumor.

6. The method of claim 1, wherein the disease is a solid tumor.

7. The method of claim 1, wherein the cells are obtained from bone marrow.

8. The method of claim 1, wherein the gene(s) is/are selected from the group consisting of *ABHD14A*, *ABO*, *ADAMTS18*, *ADRA2B*, *ADRB3*, *AIRE*, *AKAP12*, *ALOX15B*, *ALS2CR11*, *AMT*, *ANKRD33*, *APC2*, *AVP*, *BHMT*, *C18orf22*, *C19orf30*, *C1orf172*, *C1orf87*, *C3orf15*, *C1QTNF6*, *C22orf27*, *C7orf16*, *C7orf41*, *CBX7*, *CCDC19*, *CCDC81*, *CD164L2*, *CDH1*, *CDKN2B*, *CHAD*, *CHRNG*, *CIDEB*, *CKMT1B*, *CKMT2*, *CLCN6*, *CLDN6*, *CLDN9*, *CNTN4*, *CPT1B*, *CRHBP*, *CXCL5*, *CYP26C1*, *CYP2E1*, *DES*, *DPYS*, *DYDC1*, *EGFL7*, *ELMO3*, *ENTPD2*, *ENTPD3*, *ESR1*, *EYA4*, *F2RL2*, *FAM57B*,

FBLN1, *FBXO2*, *FKBP1B*, *FLJ44881*, *FLVCR2*, *FREQ*, *FZD9*, *GAB1*, *GAS2L2*, *GATA4*, *GBGT1*, *GDF5*, *GHSR*, *GNAS*, *GNMT*, *GNPNAT1*, *GP1BA*, *GPR25*, *GRM6*, *GSTM5*, *HCN4*, *HIST1H1A*, *HOXD4*, *HSPA2*, *HTATIP2*, *HTR7*, *HYDIN*, *IGDCC3*, *ILDR1*, *IRF6*, *KAZALD1*, *KCNA6*, *KCNK3*, *KCNQ1*, *KIAA0427*, *KIR3DX1*, *KRT25*, *KRT7*, *KRT72*, *LAD1*, *LAMA4*, *LAMC2*, *LGTN*, *LRRC17*, *LTF*, *MBD3L1*, *MEGF10*, *MICAL1*, *MRPL28*, *MTMR9*, *MTNR1B*, *NALCN*, *NCAN*, *NCOR2*, *NDRG2*, *NDUFAF3*, *NEUROG1*, *NGB*, *NPFFR2*, *NPM2*, *NPPB*, *NPR2*, *NXN*, *OBFC2B*, *OGFR*, *ONECUT2*, *OTOP1*, *OXT*, *PACSIN1*, *PAOX*, *PARP3*, *PAX1*, *PCDH8*, *PCDHAC2*, *PDE4C*, *PF4V1*, *PKDREJ*, *PM20D1*, *POMC*, *POU3F1*, *PPAPDC3*, *PRIC285*, *PRLH*, *PSMD11*, *PTGIS*, *RAB36*, *RAP1GAP*, *RASGRF1*, *RASIP1*, *RBPJL*, *RLN1*, *RPL36*, *RPL36AL*, *RPUSD3*, *SCG5*, *SCMH1*, *SCUBE3*, *SEMA3B*, *SGPP2*, *SHROOM1*, *SKAP1*, *SLC12A8*, *SLC5A8*, *SNN*, *SORBS3*, *SPG7*, *SPINT1*, *SRD5A2*, *SRRT*, *SSTR4*, *STMN1*, *TBC1D1*, *TCEA2*, *TCF15*, *TFAP2E*, *TGFBI*, *TIAM1*, *TMEM125*, *TMEM151A*, *TMEM184A*, *TMEM189*, *TMOD3*, *TNNT1*, *TP53INP1*, *TRPC4*, *TRPM3*, *UNC80*, *VAMP5*, *VHL*, *VSTM1*, *WBSCR27*, *WDR52*, *WT1*, *ZFP41*, *ZNF205*, and *ZNF710*.

9. The method of claim 1, wherein the gene(s) is/are selected from the group consisting of *WT1*, *CDH1*, and *CDKN2B*.

10. The method of claim 1, wherein the gene is *WT1*.

11. The method of claim 1, wherein the methylation pattern corresponds to a methylation signature provided in Figure 6.

12. The method of claim 1, wherein the therapeutic agent is a cytidine analog.

13. The method of claim 1, wherein the therapeutic agent is 5-azacytidine.

14. The method of claim 1, wherein the therapeutic agent is administered parenterally.

15. The method of claim 1, wherein the therapeutic agent is administered orally.

16. The method of claim 1, wherein the therapeutic agent is administered in a therapeutically effective amount.

17. The method of claim 16, wherein the therapeutically effective amount is between about 50 mg/m^2 and about 200 mg/m^2 per day.

18. The method of claim 17, wherein the therapeutic agent is administered parenterally.

19. A method for identifying a patient diagnosed with a myelodysplastic syndrome having an increased probability of obtaining improved overall survival following 5-azacytidine treatment.

20. The method of claim 19, which comprises analyzing methylation levels of the patient's nucleic acid.

21. The method of claim 20, wherein the nucleic acid is DNA.

22. The method of claim 20, wherein the nucleic acid is RNA.

23. The method of claim 20, which comprises analyzing the methylation level of a gene selected from the group consisting of *ABHD14A*, *ABO*, *ADAMTS18*, *ADRA2B*, *ADRB3*, *AIRE*, *AKAP12*, *ALOX15B*, *ALS2CR11*, *AMT*, *ANKRD33*, *APC2*, *AVP*, *BHMT*, *C18orf22*, *C19orf30*, *C1orf172*, *C1orf87*, *C3orf15*, *C1QTNF6*, *C22orf27*, *C7orf16*, *C7orf41*, *CBX7*, *CCDC19*, *CCDC81*, *CD164L2*, *CDH1*, *CDKN2B*, *CHAD*, *CHRNG*, *CIDEB*, *CKMT1B*, *CKMT2*, *CLCN6*, *CLDN6*, *CLDN9*, *CNTN4*, *CPT1B*, *CRHBP*, *CXCL5*, *CYP26C1*, *CYP2E1*, *DES*, *DPYS*, *DYDC1*, *EGFL7*, *ELMO3*, *ENTPD2*, *ENTPD3*, *ESR1*, *EYA4*, *F2RL2*, *FAM57B*, *FBLN1*, *FBXO2*, *FKBP1B*, *FLJ44881*, *FLVCR2*, *FREQ*, *FZD9*, *GAB1*, *GAS2L2*, *GATA4*, *GBGT1*, *GDF5*, *GHSR*, *GNAS*, *GNMT*, *GNPNAT1*, *GP1BA*, *GPR25*, *GRM6*, *GSTM5*, *HCN4*, *HIST1H1A*, *HOXD4*, *HSPA2*, *HTATIP2*, *HTR7*, *HYDIN*, *IGDCC3*, *ILDR1*, *IRF6*, *KAZALD1*, *KCNA6*, *KCNK3*, *KCNQ1*, *KIAA0427*, *KIR3DX1*, *KRT25*, *KRT7*, *KRT72*, *LAD1*, *LAMA4*, *LAMC2*, *LGTN*, *LRRC17*, *LTF*, *MBD3L1*, *MEGF10*, *MICAL1*, *MRPL28*, *MTMR9*, *MTNR1B*, *NALCN*, *NCAN*, *NCOR2*, *NDRG2*, *NDUFAF3*, *NEUROG1*, *NGB*,

NPFFR2, *NPM2*, *NPPB*, *NPR2*, *NXN*, *OBFC2B*, *OGFR*, *ONECUT2*, *OTOP1*, *OXT*, *PACSIN1*, *PAOX*, *PARP3*, *PAX1*, *PCDH8*, *PCDHAC2*, *PDE4C*, *PF4V1*, *PKDREJ*, *PM20D1*, *POMC*, *POU3F1*, *PPAPDC3*, *PRIC285*, *PRLH*, *PSMD11*, *PTGIS*, *RAB36*, *RAP1GAP*, *RASGRF1*, *RASIP1*, *RBPJL*, *RLN1*, *RPL36*, *RPL36AL*, *RPUSD3*, *SCG5*, *SCMH1*, *SCUBE3*, *SEMA3B*, *SGPP2*, *SHROOM1*, *SKAP1*, *SLC12A8*, *SLC5A8*, *SNN*, *SORBS3*, *SPG7*, *SPINT1*, *SRD5A2*, *SRRT*, *SSTR4*, *STMN1*, *TBC1D1*, *TCEA2*, *TCF15*, *TFAP2E*, *TGFBI*, *TIAM1*, *TMEM125*, *TMEM151A*, *TMEM184A*, *TMEM189*, *TMOD3*, *TNNT1*, *TP53INP1*, *TRPC4*, *TRPM3*, *UNC80*, *VAMP5*, *VHL*, *VSTM1*, *WBSCR27*, *WDR52*, *WT1*, *ZFP41*, *ZNF205*, and *ZNF710*.

24. The method of claim 20, which comprises analyzing the methylation level of a gene selected from the group consisting of *WT1*, *CDH1*, and *CDKN2B*.

25. The method of claim 20, which comprises analyzing the methylation level of a gene, which is *WT1*.

26. The method of claim 20, in which the patient's increased probability of obtaining improved overall survival following 5-azacytidine treatment is used to plan or adjust the patient's 5-azacytidine treatment.

27. The method of claim 19, in which the increased probability is a 10% greater probability, a 50% greater probability, a 100% greater probability, or a 200% greater probability.

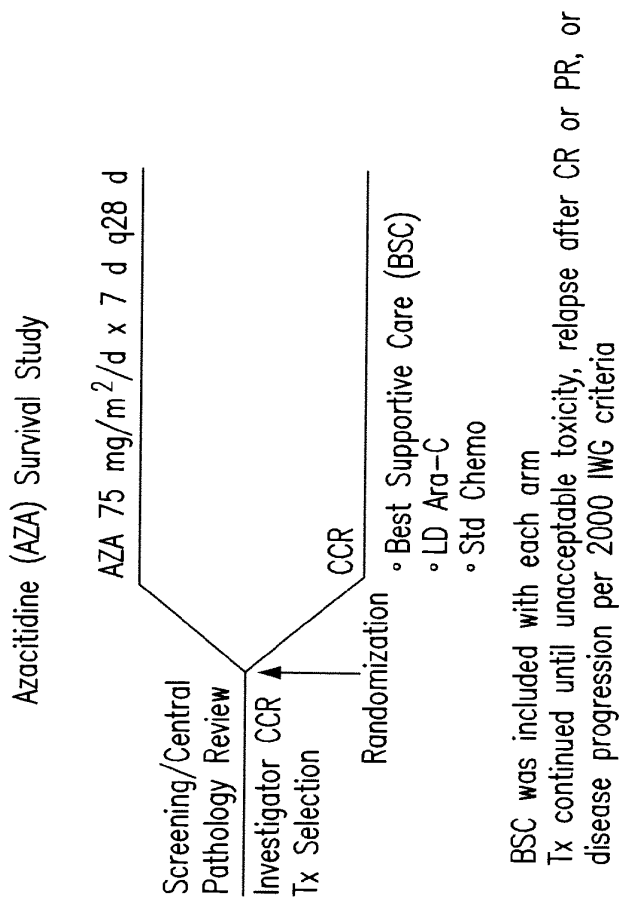


FIG. 1

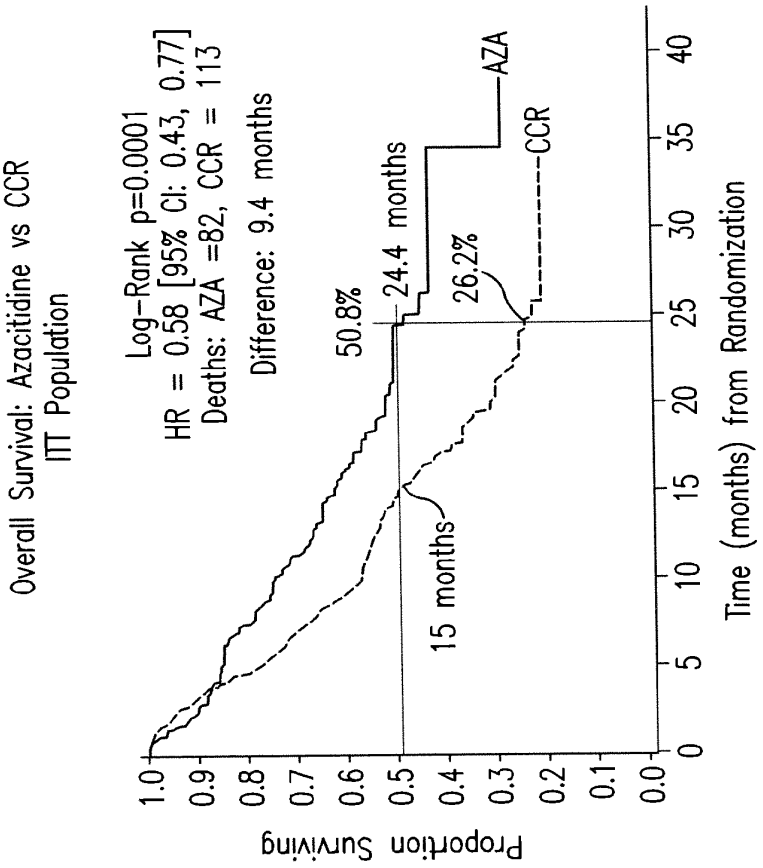


FIG. 2

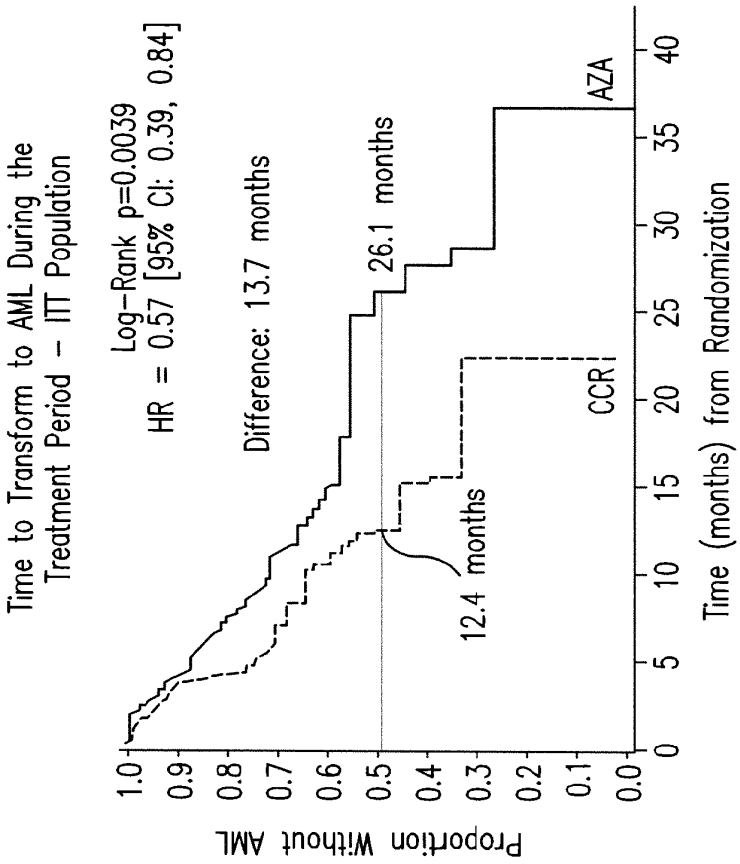


FIG. 3

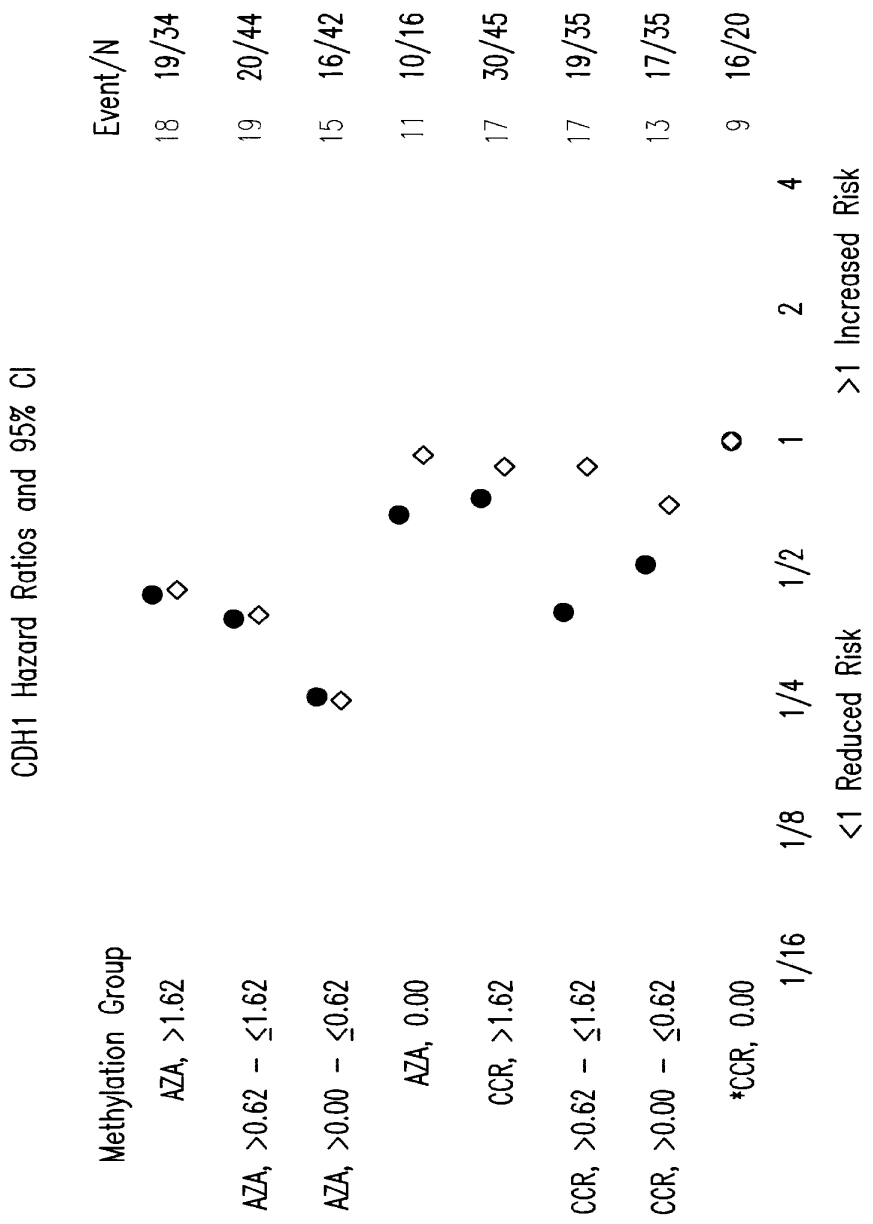


FIG. 4

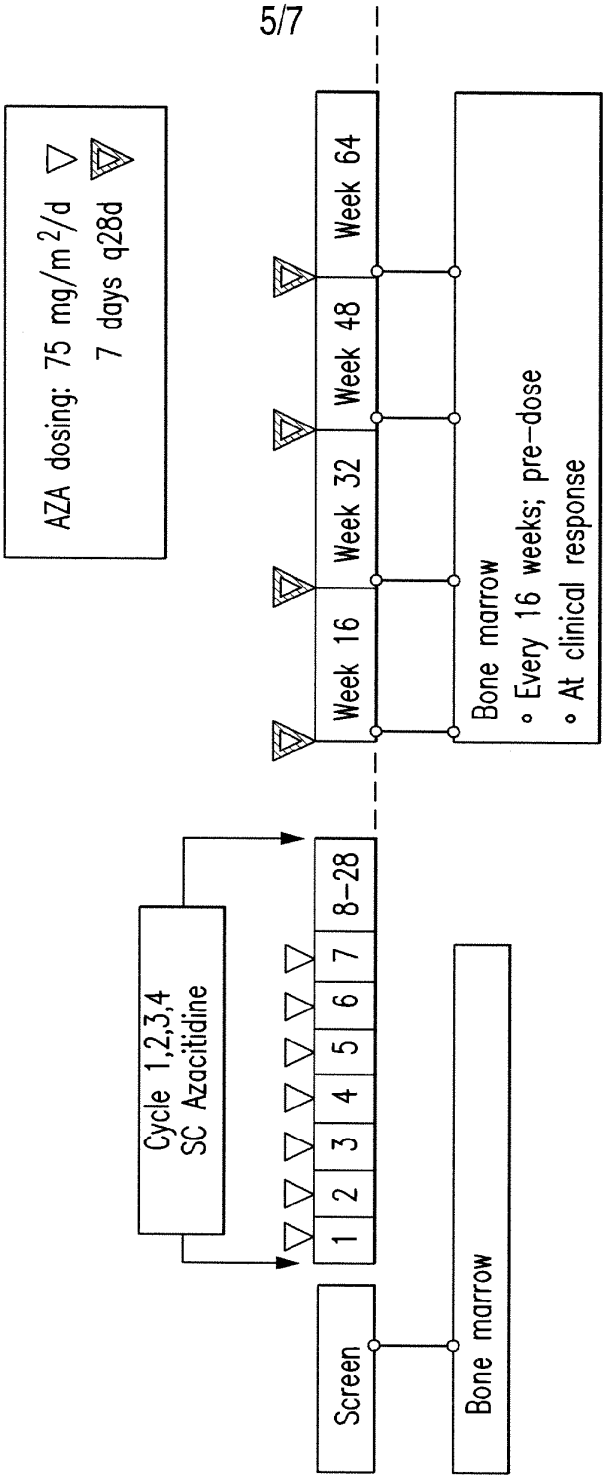


FIG. 5

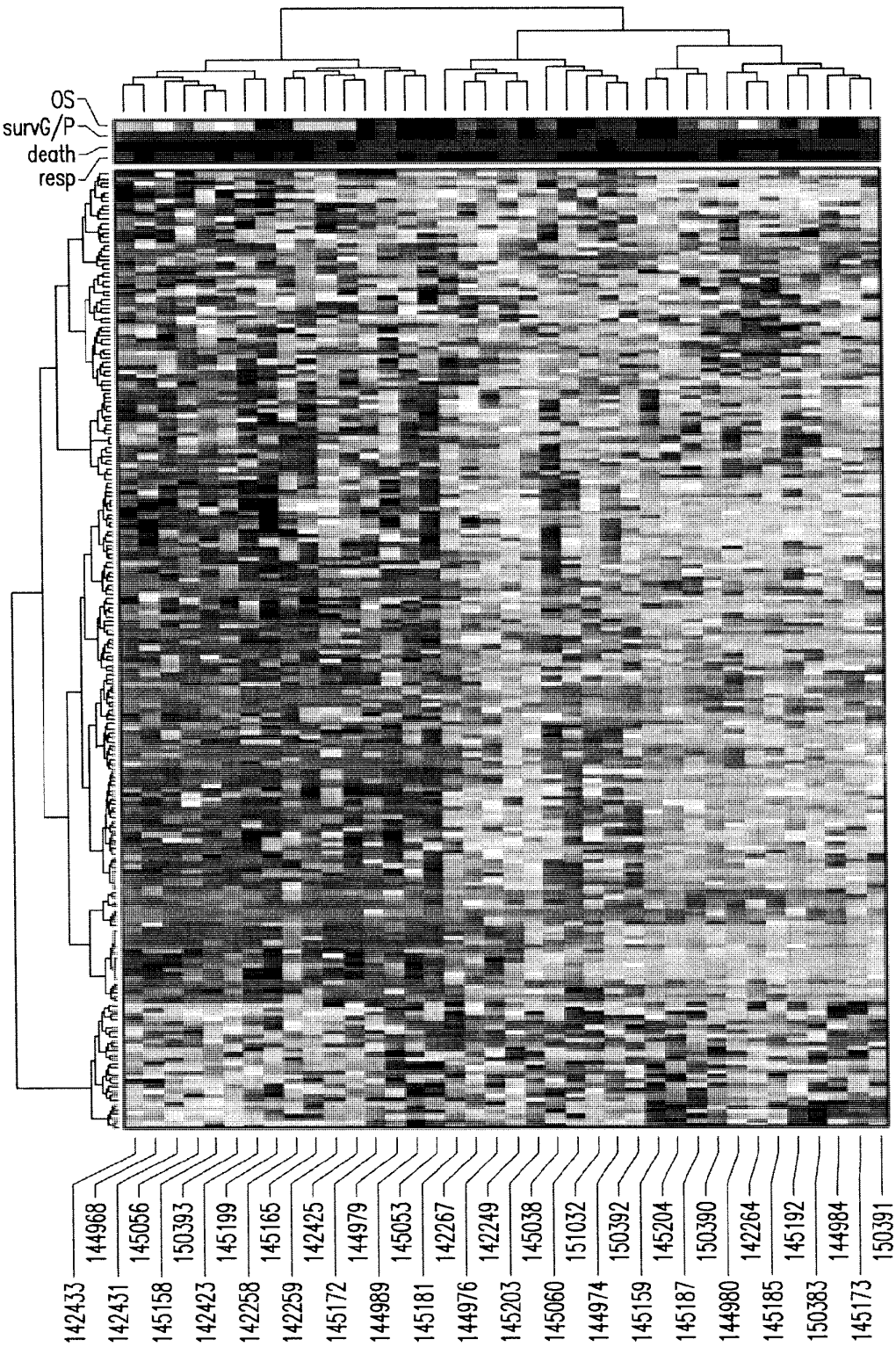
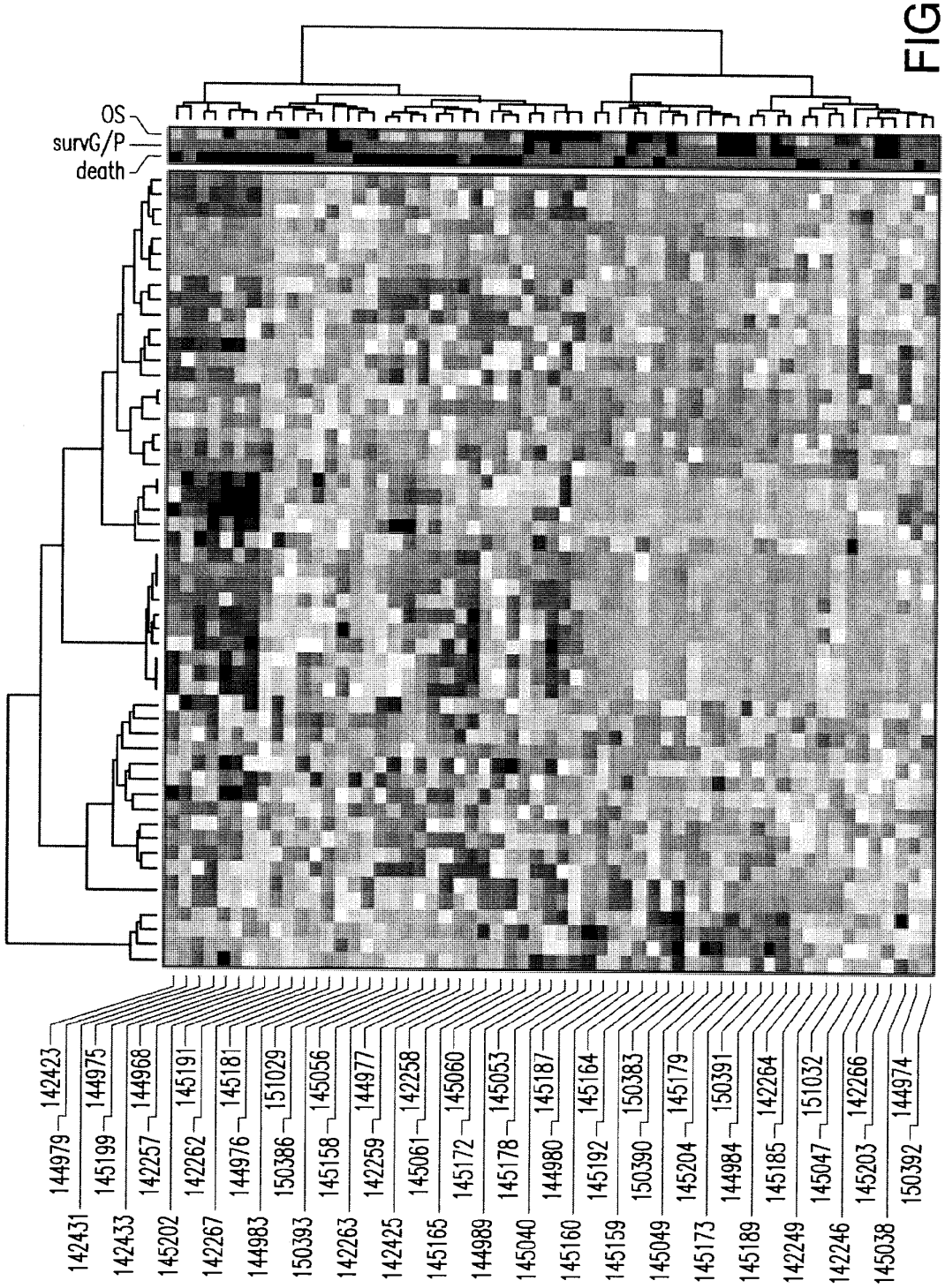


FIG. 6

FIG. 7



INTERNATIONAL SEARCH REPORT

International application No
PCT/US2012/049826

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, Sequence Search, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2009/058394 A1 (CELGENE CORP [US]; BEACH C L [US]; BACKSTROM JAY THOMAS [US]) 7 May 2009 (2009-05-07) claims 22-32	1-27

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 September 2012	10/01/2013

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Dolce, Luca

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2012/049826

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-27(partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2012/049826

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MACBETH KYLE J ET AL: "A Comparative Pharmacokinetic/Pharmacodynamic (PK/PD) Evaluation of Azacitidine Following Subcutaneous (SC) and Oral Administration in Subjects with Myelodysplastic Syndromes (MDS) or Acute Myelogenous Leukemia (AML), Results From a Phase 1 Study", BLOOD, vol. 114, no. 22, November 2009 (2009-11), page 705, XP008155862, & 51ST ANNUAL MEETING OF THE AMERICAN-SOCIETY-OF-HEMATOLOGY; NEW ORLEANS, LA, USA; DECEMBER 05 -08, 2009 the whole document ----- BYUN HYANG-MIN ET AL: "Genome-Wide DNA Methylation Analysis of Patients with Myelodysplastic Syndrome After Azacitidine Treatment", BLOOD, vol. 114, no. 22, November 2009 (2009-11), page 249, XP008155852, & 51ST ANNUAL MEETING OF THE AMERICAN-SOCIETY-OF-HEMATOLOGY; NEW ORLEANS, LA, USA; DECEMBER 05 -08, 2009 the whole document ----- FABIANI EMILIANO ET AL: "Analysis of genome-wide methylation and gene expression induced by 5-aza-2'-deoxycytidine identifies BCL2L10 as a frequent methylation target in acute myeloid leukemia.", LEUKEMIA & LYMPHOMA DEC 2010 LNKD-PUBMED:21077739, vol. 51, no. 12, December 2010 (2010-12), pages 2275-2284, XP008155856, ISSN: 1029-2403 the whole document ----- HOSHINO JUN ET AL: "Dorz1, a novel gene expressed in differentiating cerebellar granule neurons, is down-regulated in Zic1-deficient mouse.", MOLECULAR BRAIN RESEARCH, vol. 120, no. 1, 12 December 2003 (2003-12-12), pages 57-64, XP055037537, ISSN: 0169-328X the whole document ----- US 2010/009364 A1 (FANTL WENDY J [US] ET AL) 14 January 2010 (2010-01-14) the whole document -----	1-27 1-27 1-27 8,23 1-27

Form PCT/ISA/210 (continuation of second sheet) (April 2005)

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-27(partially)

methods to predict the sensibility/response of a patient suffering from myelosdysplastic syndrome (MDS) to 5-azacytidine (AZA) based on evaluating the methylation status of ABHD14A; use of AZA to treat a subgroup of MDS patients with an increased likelihood of response, as identified by analysing the methylation level of ABHD14A

2-190. claims: 1-27(partially)

same as above for all remaining genes mentioned in claim 8.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2012/049826

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2009058394 A1	07-05-2009	CA 2742252 A1	07-05-2009
		EP 2211870 A1	04-08-2010
		JP 2011505336 A	24-02-2011
		US 2010311683 A1	09-12-2010
		WO 2009058394 A1	07-05-2009
US 2010009364 A1	14-01-2010	EP 2304436 A1	06-04-2011
		US 2010009364 A1	14-01-2010
		WO 2010006291 A1	14-01-2010

View of NCT00748553 on 2011_08_05

ClinicalTrials Identifier: NCT00748553
Updated: 2011_08_05

Descriptive Information

Brief title
A Phase I/II Clinical Trial of Vidaza With Abraxane in the Treatment of Patients With Advanced or Metastatic Solid Tumors and Breast Cancer

Official title
A Phase I/II Clinical Trial of the Hypomethylating Agent Azacitidine (Vidaza) With the Nanoparticle Albumin Bound Paclitaxel (Abraxane) in the Treatment of Patients With Advanced or Metastatic Solid Tumors and Breast Cancer

Brief summary

The purpose of this clinical trial is to test whether treatment of patients with advanced or metastatic solid tumors or breast cancer with Abraxane plus Vidaza is safe and results in good tumor response. All patients enrolling in this study will receive treatment with Abraxane and Vidaza. Safety will be assessed by adverse events, laboratory results and performance status. Tumor response will be measured by RECIST criteria.

Detailed description

The phase I part of the study will enroll patients with advanced or metastatic solid tumors who have failed at least one previous treatment. The purpose of the phase I part is to assess the safety of the investigational treatment and select the recommended phase II dose-regimen. The phase II part of the study will enroll patients with advanced or metastatic HER2-negative breast cancer who have not received treatment for their metastatic disease. The purpose of the phase II part of the study is to assess safety and efficacy of the investigational treatment in breast cancer. The study doctor will determine what phase patients will be enrolled in.

Phase	Phase 1
Phase	Phase 2
Study type	Interventional
Study design	Treatment
Study design	Non-Randomized
Study design	Open Label
Study design	Single Group Assignment
Study design	Safety/Efficacy Study
Primary outcome	Measure: Phase I: To assess safety of Vidaza and Abraxane combination and to select the recommended phase II dose-regimen Time Frame: 6 months Safety Issue? Yes
Primary outcome	Measure: Phase II: To assess clinical efficacy of Vidaza and Abraxane combination Time Frame: 1.5 years Safety Issue? Yes

BNSDOCID: <XP_____5505186BA__I_>

Secondary outcome	Measure: To explore the relationship between specific biomarkers and cancer- and treatment-related outcomes; Time Frame: 2 years Safety Issue? No
Secondary outcome	Measure: To assess progression-free survival. Time Frame: 2 years Safety Issue? No
Condition	Advanced or Metastatic Solid Tumors
Condition	Advanced or Metastatic Breast Cancer
Intervention	Drug: Azacitidine (Vidaza) 50mg/m2, 75mg/m2 or 100mg/m2 daily for 5 days for each 4-week cycle
Intervention	Drug: Nab-paclitaxel (Abraxane) 100mg/m2 weekly for 3 weeks of each 4-week cycle
URL	http://www.southalabama.edu/mci/
See also	USA Mitchell Cancer Institute website

Recruitment Information

Status	Recruiting
Start date	2008-09
Last follow-up date	2012-09 (Anticipated)
Primary completion date	2012-09 (Anticipated)

Criteria

Inclusion Criteria:

1. For phase I, any solid tumors, including lymphoma, that progressed or were stable as best response on at least one previous therapy and are evaluable.
2. For phase II, pathologically confirmed breast cancer, measurable disease, no prior treatments for recurrent or metastatic breast cancer.
3. Her-2/neu negative (Phase II)
4. Negative pregnancy test for female subjects
5. Women of childbearing potential should be advised to avoid becoming pregnant and men should be advised to not father a child while receiving treatment with azacitidine or nab-paclitaxel. investigator.
6. Male or female for phase I and female for phase II, >19 years of age and any race.

Exclusion Criteria:

1. Major surgery, radiotherapy, chemotherapy or investigational agents within 4 weeks of treatment day 1
2. Known brain or leptomeningeal metastases
3. Prior taxanes (except for adjuvant therapy more than 6 months prior to treatment day 1) (phase II)
4. Active infection requiring antibiotic therapy
5. History of allergy or hypersensitivity to nab-paclitaxel, albumin or a taxane
6. Grade 2 or greater motor or sensory neuropathy
7. Prior cytotoxic chemotherapy for recurrent or metastatic breast cancer (phase II portion)
8. Uncontrolled hypertension, arrhythmia, congestive heart failure or angina. Patients who have had a myocardial infarction or cardiac surgery should be at least 6 months from the event and free of active symptoms.

9. Known or suspected hypersensitivity to azacitidine or mannitol
10. Pregnant or breast feeding
11. Patients with advanced malignant hepatic tumors
12. Malignancy other than breast carcinoma (phase II)
13. Known HIV infection or chronic hepatitis B or C

Gender	Both
Minimum age	19 Years
Healthy volunteers	No

Administrative Data

Organization name	University of South Alabama
Organization study ID	VAST-B
Sponsor	University of South Alabama
Collaborator	Abraxis BioScience, LLC.
Collaborator	Celgene Corporation
Health Authority	United States: Institutional Review Board

View of NCT01478685 on 2011_11_22

ClinicalTrials Identifier: NCT01478685
Updated: 2011_11_22

Descriptive Information

Brief title Oral Azacitidine as a Single Agent and in Combination With Carboplatin or Abraxane® in Subjects With Relapsed or Refractory Solid Tumors

Official title A Phase I Study of Oral Azacitidine as a Single Agent and in Combination With Carboplatin or Abraxane® in Subjects With Relapsed or Refractory Solid Tumors

Brief summary

The purpose of the study is to evaluate the safety and to define the Maximal Tolerated Dose (MTD) or the Maximal Administered Dose (MAD) of oral azacitidine as a single agent and in combination with carboplatin (CBDCA) or paclitaxel protein bound particles (Abraxene® [ABX]) in subjects with relapsed or refractory solid tumors.

Detailed description

Phase Phase 1
Study type Interventional
Study design Treatment
Study design Non-Randomized
Study design Open Label
Study design Parallel Assignment
Study design Safety Study

Primary outcome
Measure: Adverse Events
Time Frame: Up to 2 years
Safety Issue? Yes
Description:

Number of participants with adverse events using the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI CTCAE) criteria, Version 4.0.

Secondary outcome
Measure: Cmax
Time Frame: Up to 30 days
Safety Issue? No
Description:

maximum observed concentration in plasma (Cmax)

Secondary outcome
Measure: AUC
Time Frame: Up to 30 days
Safety Issue? No
Description:

	area under the concentration-time curve (AUC)
Secondary outcome	Measure: tmax Time Frame: Up to 30 days Safety Issue? No Description:
	time to maximum concentration (tmax);
Secondary outcome	Measure: (t1/2) Time Frame: Up to 30 days Safety Issue? No Description:
	terminal half-life (t1/2)
Secondary outcome	Measure: (CL/F) Time Frame: Up to 30 days Safety Issue? No Description:
	apparent total body clearance (CL/F)
Secondary outcome	Measure: (Vz/F) Time Frame: Up to 30 days Safety Issue? No Description:
	apparent volume of distribution (Vz/F).
Secondary outcome	Measure: DNA Methylation Time Frame: Up to 30 days Safety Issue? No Description:
	Change from baseline (Cycle 1 Day 1 pre-dose) in DNA methylation (global and gene-specific assays) in whole blood and tumor tissue (as available in Part 1)
Secondary outcome	Measure: DNMT1 protein levels Time Frame: Up to 30 days Safety Issue? No Description:
	Reduction from baseline (Cycle 1 Day 1 predose) in DNMT1 protein levels in tumor tissue (as available in Part 1)
Secondary outcome	Measure: Tumor response rate Time Frame: Up to 2 years Safety Issue? No Description:
	Tumor response rate
Secondary outcome	Measure: Number of participants who survive without disease progression Time Frame: Up to 2 years Safety Issue? No Description:
	Number of participants who survive without disease progression

Condition	Relapsed or Refractory Solid Tumors
Arm/Group	Arm Label: Oral Azacitidine - Single agent Experimental
Arm/Group	Arm Label: Oral azacitidine plus carboplatin Experimental
Arm/Group	Arm Label: Oral azacitidine plus Abraxane Experimental
Intervention	Drug: Oral azacitidine Arm Label: Oral Azacitidine - Single agent Oral azacitidine will be administered at doses between 100-300 mg daily for either 14 or 21 days depending on tolerability.
Intervention	Drug: Carboplatin Arm Label: Oral azacitidine plus carboplatin Carboplatin will be given on Day 1 of every 21 days at a dosage of area under the curve times 4.
Intervention	Drug: Abraxane Arm Label: Oral azacitidine plus Abraxane Abraxane will be given weekly at a dosage of 100 mg/m2.

Recruitment Information

Status	Recruiting
Start date	2011-11
Last follow-up date	2014-01 (Anticipated)
Primary completion date	2013-11 (Anticipated)

Criteria

Inclusion Criteria:

1. Men and women, 18 years or older at the time of signing the Informed Consent Document (ICD).
2. Understand and voluntarily sign an ICD prior to any study-related assessments or procedures are conducted.
3. Able to adhere to the study visit schedule and other protocol requirements.
4. With histological or cytological confirmation of advanced unresectable solid tumors, including those who have progressed on (or not been able to tolerate) standard anticancer therapy, or for whom no other effective therapy exists, or for who declines standard therapy.
5. Consent to screening tumor biopsy (for accessible tumors when appropriate [optional in Part 1, mandatory in Part 2]).
6. Eastern Cooperative Oncology Group (ECOG) Performance Status of ≤ 2.
7. The following laboratory values:
- Absolute neutrophil count (ANC) ≥ 1.5 X 109/L
- Hemoglobin (Hgb) ≥90 g/L
- Platelets (plt) ≥ 100 x 109/L
- Potassium within normal range, or correctable with supplements;
- AST and ALT ≤2.5 x Upper Limit Normal (ULN) or ≤5.0 x ULN if liver tumor is present;
- Serum total bilirubin ≤ 1.5 x ULN
- Serum creatinine ≤ 1.5 x ULN, or 24-hr clearance ≥ 60ml/min; and
- Negative serum pregnancy test within 7 days before starting study treatment in females of childbearing potential (FCBP)
8. Part 2 only: Measureable tumor (types to be determined during Part 1)
9. Females of child-bearing potential {defined as a sexually mature women who
- has not undergone a hysterectomy (the surgical removal of the uterus) or bilateral oophorectomy (the surgical removal of both ovaries) or,
- has not been naturally postmenopausal for at least 24 consecutive months (i.e., has had menses at any time during the preceding 24 consecutive months} must

-- agree to the use of a physician- approved contraceptive method (oral, injectable, or implantable hormonal contraceptive ; tubal ligation; intra-uterine device; barrier contraceptive with spermicide; or vasectomized partner) while on oral azacitidine and for 3 months following the last dose of study medication; and
-- have a negative serum pregnancy test during screening
10. Male subjects with female partner of childbearing potential must agree to the use of a physician-approved contraceptive method throughout the course of the study to avoid fathering a child during the course of the study and for 6 months following the last dose of oral azacitidine.

Exclusion Criteria:
1. Any significant medical condition, laboratory abnormalities, which places the subject at unacceptable risk if he/she were to participate in the study.
2. Any condition that confounds the ability to interpret data from the study.
3. Symptomatic central nervous system metastases. Subjects with brain metastases that have been previously treated and are stable for 6 weeks are allowed.
4. Known acute or chronic pancreatitis.
5. Any peripheral neuropathy ≥ NCI CTCAE grade 2.
6. Persistent diarrhea or malabsorption ≥ NCI CTCAE grade 2, despite medical management.
7. Impaired ability to swallow oral medication.
8. Unstable angina, significant cardiac arrythmia, or New York Heart Association (NYHA) class 3 or 4 congestive heart failure.
9. Prior systemic cancer-directed treatments or investigational modalities ≤ 5 half lives or 4 weeks, whichever is shorter, prior to starting study drug or who have not recovered from side effects of such therapy. (except alopecia).
10. Major surgery ≤ 2 weeks prior to starting a study drug or who have not recovered from side effects of such therapy.
11. Pregnant or breast feeding.
12. Known Human Immunodeficiency Virus (HIV) infection.
13. Known chronic hepatitis B or C virus (HBV/HCV) infection, unless this is a comorbidity in subjects with HCV.

Gender	Both
Minimum age	18 Years
Healthy volunteers	No

Administrative Data

Organization name	Celgene Corporation
Organization study ID	AZA-ST-001
Sponsor	Celgene Corporation
Health Authority	United States: Food and Drug Administration

ASCO American Society of Clinical Oncology
www.asco.org

A phase IIa study of a sequential regimen using azacitidine to reverse platinum resistance to carboplatin in patients with platinum resistant or refractory epithelial ovarian cancer.

Sub-category:
Epigentic Strategies

Category:
Developmental Therapeutics - Experimental Therapeutics

Meeting:
2008 ASCO Annual Meeting

Session Type and Session Title:
Clinical Science Symposium, Epigenetic Approaches to Cancer Treatment: Strategies and Issues

Abstract No:
3500

Citation:
J Clin Oncol 26: 2008 (May 20 suppl; abstr 3500)

Author(s):
R. C. Bast, R. B. Iyer, W. Hu, J. Kavanagh, R. L. Coleman, C. Levenback, A. K. Sood, J. Wolf, D. M. Gershenson, M. Markman, S. Fu

Abstract Disclosures

Abstract:

Background: Our preclinical model established the rationale for using azacitidine to reverse platinum resistance. Sequential azacitidine and carboplatin treatment conferred an effect against platinum resistant ovarian cancer cells that was greater than from each agent alone (synergistic interaction). Based on a well tolerated phase I regimen of azacitidine 75 mg/m^2/day for 5 days plus carboplatin AUC 5 on day 2 once every 28 days, we initiated a phase IIa trial to evaluate efficacy. **Methods:** Patients with pathologically confirmed epithelial ovarian cancer who progressed within 6 months after a platinum-based regimen (resistant, Plat-RS, n=18) or on a platinum- based regimen (refractory, Plat-RF, n=12), were eligible to receive sequential azacitidine and carboplatin. All patients had measurable disease. **Results:** Thirty patients received a total of 159 cycles of treatment and 2 patients are still being actively treated at the cycle 14 and 19, respectively. Side effects include neutropenia, anemia, fatigue, nausea, and pain/irritation at the injection sites. All patients were followed for more than 12 months or until death. Remarkably, our regimen resulted in 1 CR, 3 PR (RR: 14%) and 10 SD in 29 evaluable patients, which is in stark contrast to no responses to a carboplatin-based regimen in a similar cohort of patients in the same institution. The median duration of response was 7.5 months. OS at 1-yr was 53%. Patients classified as having Plat-RS disease had an objective response of 22% and one-year OS of 67% compared to 0% and 33% in Plat-RF patients, respectively. **Conclusions:** To the best of our knowledge, this is the first phase II trial in epithelial ovarian cancer patients that demonstrates the ability of a hypomethylating agent to reverse platinum resistance. Based on these data, a randomized phase III trial in patients with platinum-resistant epithelial ovarian cancer is warranted.

Associated Presentation(s):

1. A phase IIa study of a sequential regimen using azacitidine to reverse platinum resistance to carboplatin in patients with platinum resistant or refractory epithelial ovarian cancer.

Will DNA methylation inhibitors work in solid tumors? A review of the clinical experience with azacitidine and decitabine in solid tumors

The recent approval of azacitidine (Vidaza®), decitabine (Dacogen®) and vorinostat (Zolinza™) for myelodysplastic syndrome and cutaneous T-cell lymphoma has led to a wave of interest in epigenetic therapy. These DNA methylation inhibitors and the histone deacetylase inhibitor clearly have demonstrated activity in hematologic malignancies, but the future role of epigenetic therapy in solid tumors is still unknown. What is not commonly known is that azacitidine and decitabine were originally developed as cytotoxic nucleoside analogs and clinical trials were previously conducted in a variety of cancer types prior to the knowledge of their ability to inhibit DNA methylation. We review the experience of azacitidine and decitabine in early clinical trials and demonstrate the activity of epigenetic therapy in solid tumors.

KEYWORDS: azacitidine cancer decitabine developmental therapeutics DNA methylation epigenetics

Issa A Cowan, Sundeep Talwar & Allen S Yang†

Epigenetics is a rapidly developing field that focuses on heritable genetic information that is not coded for in the sequence of the DNA. The vast majority of epigenetic research focuses on DNA methylation, histone modifications and chromatin structure [1,2]. The field has been catapulted to the forefront of cancer research owing to the recent approval of three drugs that attack cancer-related changes to the epigenome: azacitidine (Vidaza®, Pharmion Corporation, CO, USA), decitabine (Dacogen®, MGI Pharma Inc., MN, USA) and vorinostat (Zolinza™, Merck & Co., Inc., NJ, USA). In addition, a multitude of next-generation epigenetic therapies are currently in development [3]. Azacitidine and decitabine exert their therapeutic effect by inhibiting DNA methylation levels [4]. Large resources are being invested into a better understanding of how the epigenome is organized in normal tissues and how the epigenome becomes altered in diseases like cancer. Recent studies demonstrate that the environment can affect the epigenome, and a rudimentary understanding is beginning to develop of how a lifetime of environmental exposure can lead to epigenetic changes that result in disease [5,6]. Eventually, an improved understanding of the aberrant alterations in the epigenome that result in disease will undoubtedly lead to improved targeted therapies to treat disease.

DNA methylation is a postreplicative chemical modification of DNA. In mammals, cytosine can be methylated to 5-methylcytosine at the palindromic CpG dinucleotide. The CpG palindrome allows DNA methyltransferases to faithfully copy the DNA methylation pattern from the parent to the complementary daughter strand of DNA. DNA methylation is associated with transcriptional silencing of genes, but the chemical methylation of DNA in itself does not inhibit transcription of mRNA. Instead, the DNA methylation tags regions of the genome to recruit methyl-binding proteins that are associated with histone-modifying enzymes, such as histone deacetylases (HDACs). DNA methylation and histone deacetylation lead to compaction of DNA tightly around histones and a compaction of chromatin leads to inaccessibility by the transcriptional machinery and transcriptional silencing. Aberrant DNA hypermethylation of normally unmethylated CpG rich areas referred to as CpG islands seems to be the most prevalent event described in cancer. Hypermethylation of the CpG islands usually associated with the promoter region of genes leads to the aberrant silencing of numerous genes including a number of tumor suppressor genes (reviewed in [7]).

Abnormal DNA methylation has been used successfully to classify different cancers, and characterize certain disease phenotypes. There are numerous reports that describe the hypermethylation of a gene being associated with prognosis for gastric, lung, esophageal, pancreatic and colon cancer (reviewed in [8]). Acute lymphoblastic leukemia and acute myeloid leukemia (AML) with hypermethylation have also been associated with a poorer outcome [9–12]. DNA methylation patterns have also been demonstrated to predict response or resistance to therapy in glioma and melanoma [13,14]. Numerous studies have

future medicine part of fsg

described abnormal DNA methylation in myelodysplastic syndrome (MDS) for various genes, and it is believed that DNA methylation inhibitor drugs therapeutically reverse this abnormal DNA methylation.

Azacitidine and decitabine are cytosine analogs and were empirically demonstrated to have clinical activity in myeloid leukemia and other cancers prior to knowledge of their activity as epigenetic therapies. Azacitidine and decitabine were used in a number of early clinical studies for both solid and hematological malignancies. The drugs are generally believed to have good clinical activity in AML. The response rate for decitabine in AML has been demonstrated to be 33–89% as a first-line or salvage-therapy single agent [15]. Unfortunately, the drugs were used at high doses at or near the maximum tolerated dose as was the practice of the time for cytotoxic chemotherapy agents. There was no knowledge of the epigenetic effects and the need for lower doses to achieve the optimal biological effect [15]. In consequence, clinical interest in azacitidine and decitabine faded before the knowledge of their ability to inhibit DNA methylation and induce differentiation became understood.

Azacitidine and decitabine have an unique chemical structure that allows them to covalently trap DNA methyltransferase and therefore suppress DNA methylation [16]. Both drugs are cytidine analogs that require conversion to 5-aza-2′deoxyazacytidine triphosphate in order to be incorporated into the DNA (Figure 1). Once in the DNA, the azacytosine ring has the unique ability to covalently trap the enzyme DNA methyltransferase and act as a suicide inhibitor of the enzyme thereby depleting the cell of active DNA methyltransferase and preventing the future modification of cytosine to 5-methylcytosine in the genome (Figure 2). It is important to note that DNA methylation is not actively removed from the DNA, but the enzyme that maintains DNA methylation is inhibited therefore requiring many subsequent cell divisions to lead to a decrease in DNA methylation [17,18]. This may in part explain why the clinical effect of DNA methylation inhibitors is often delayed [19].

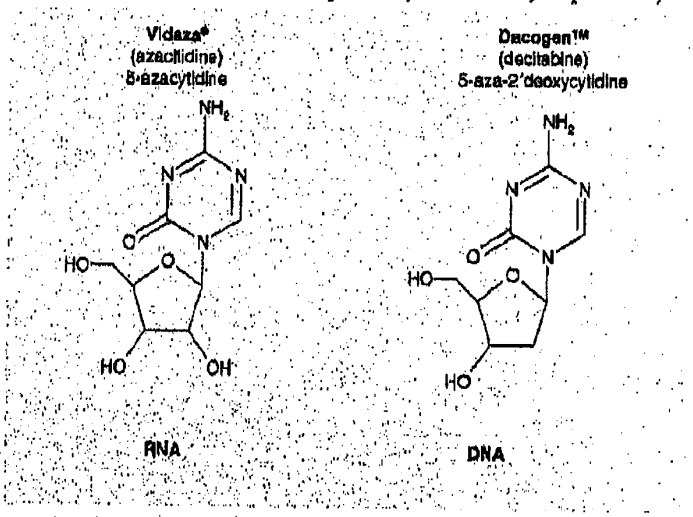


Figure 1. Azacitidine versus decitabine. The chemical structures of azacitidine (Vidaza®) a ribose and RNA precursor and decitabine (Dacogen®) a DNA precursor.

Decitabine, 5-aza-2′deoxyazacytidine, is a deoxyribonucleotide and is phosphorylated to a triphosphate form that is directly incorporated into the DNA to inhibit DNA methylation. By contrast, azacitidine, or 5-azacytidine, is a ribonucleic acid precursor and only a fraction of azacitidine is eventually reduced by ribonucleotide reductase to a deoxyribonucleic acid prior to incorporating into DNA (Figure 1) [20]. *In vitro* studies have demonstrated that azacitidine has only about 10% the potency of decitabine at inhibiting the DNA methylation [17]. Several other drugs have been demonstrated to inhibit DNA methylation. 5-fluorodeoxycytidine, 5,6-dihydro-5-azacytidine (DHAC), and more recently zebularine are pyrimidine analogs that incorporate into DNA and covalently trap DNA methyltransferase like azacitidine and decitabine [21–23]. In addition S-110 [24] and 2′-deoxy-N4-(2-(4-nitrophenyl) ethoxycarbonyl)-5-azacytidine (NPEOC-DAC) [25] are modified forms of decitabine that act as prodrugs of decitabine. Numerous agents that are not cytidine analogs have been demonstrated to inhibit DNA methylation, and are currently in development [26–36]. Although the potency of non-nucleotide analogs has been questioned [33].

Silverman and colleagues should be given credit for translating the basic chemical understanding of how DNA methylation inhibitors can be optimized to treat cancer. In Silverman's Cancer and Leukemia Group B (CALGB) 9221 study, a clear clinical benefit was demonstrated for azacitidine in MDS, and equally important, it was found that DNA methylation inhibitors were slow acting and took several courses of treatment before a clinical benefit could be seen [19]. In addition, the clinical benefit was often control, rather than eradication of the disease. These are all of the characteristics that fit with the passive loss of DNA methylation that are observed with DNA methylation inhibitors. Owing to the design of the 9221 study, a clear survival benefit

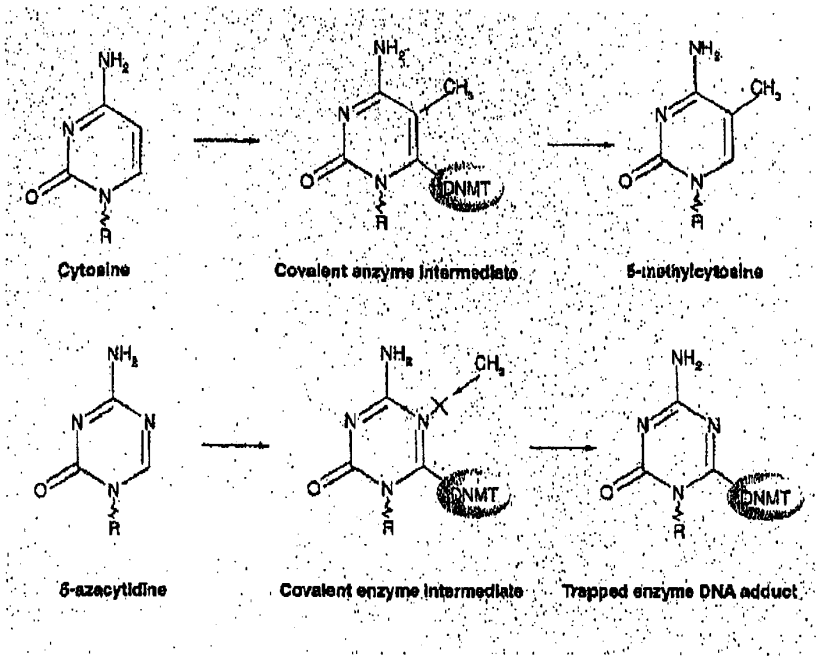


Figure 2. DNA methylation and DNA methyltransferase inhibition. The normal chemical mechanism by which DNMT methylates cytosine to 5-methylcytosine is shown above. The mechanism by which 5-azacytidine (R: either ribose [azacitidine] or deoxyribose [decitabine]) irreversibly inhibits DNMT and prevents DNA methylation.
DNMT: DNA methyltransferase.

could not be demonstrated; however, a follow-up randomized study proved that azacitidine was superior to supportive care, low-dose cytarabine, and intensive chemotherapy [37]. Interestingly, the survival benefit was seen in patients who did not achieve a complete remission, the traditional surrogate marker for survival cytotoxic therapies in hematologic malignancies, and may reflect the unique biological mechanism of azacitidine. Decitabine has also demonstrated a clear benefit in patients, but a clear survival benefit in MDS patients has yet to be demonstrated. This is probably owing to suboptimal dosing and schedules used in clinical studies to date [38–40].

Following the success of azacitidine and decitabine in MDS, the purpose of this study is to explore the possibility of whether azacitidine and decitabine will have clinical activity in solid tumors. Clearly, DNA methylation inhibitors have been successful against MDS and will likely be successful against other hematologic malignancies such as AML. Ongoing discussion about the activity of DNA methylation inhibitors against solid tumors supports that stably altering gene expression would have transformative potential in cancer therapy [41–46]. There is ample biological rationale for the activity of epigenetic therapy against solid tumors owing the abundance of epigenetic aberrations described in solid cancers. However, this work focuses on the empiric evidence that DNA methylation inhibitors are active in solid organ cancers and not just the blood system. We will therefore intentionally be agnostic to the mechanism of action of azacitidine and decitabine in attempting to answer this question. We have carefully and systematically reviewed clinical trials of azacitidine and decitabine that have focused on nonhematologic malignancies. In addition, we have chosen to review only clinical studies that use azacitidine and decitabine as a single agent and not in combination with other agents to better define the true clinical activity of DNA methylation inhibitors in solid organ cancers.

Methods & data collection

We identified all clinical studies available in the medical literature database, Pubmed, in July, 2009, that used either azacitidine or decitabine as a single agent in treatment against solid tumors. In total, 11 Phase I or II studies using azacitidine (694 total patients treated, 573 solid tumor patients used in this analysis) and eight studies using decitabine (256 total patients treated,

and 223 solid tumor patients analyzed) were reviewed for our analysis. Data collected from these studies included the number of patients, types of cancer treated, response, schedule and dose of DNA methylation inhibitor studied. The individual responses reported in each study were categorized as response (decrease in tumor size), stable disease or progression (increase in tumor size). One study using decitabine as a single agent to treat advanced solid tumors could not be included in this analysis because the stable disease rate was not reported [47]. The study observed one partial response in a patient with undifferentiated carcinoma of the ethmoid sinus, and an unspecified number of short-lasting disease stabilizations out of a total of 21 patients. The data collected used in this analysis is provided as an Excel spreadsheet (Online supplementary data, www.futuremedicine.com/doi/suppl/10.2217/epi.09.44/suppl_file/suppl_table_1.xls).

In order to ascertain whether or not there is a difference in response and stable disease rate at different doses of azacitidine, we conducted Fisher's Exact test to determine the statistical significance between the response and stable rate in patients treated with a 67.5 mg/m^2/day azacitidine dose versus all other azacitidine doses. P-values for this dose comparison were calculated from gastrointestinal cancer, breast cancer, lung cancer, and melanoma patient response and stable disease data. An identical calculation was completed to distinguish patient response to different routes of administration. Data for subcutaneous and intravenous drug administration were analyzed using the Fisher's Exact test to evaluate the significance of differences in patient response. P-values of less than 0.05 are considered significant.

Results

Azacitidine & decitabine as a single agent in solid tumors

The clinical activity of azacitidine in solid tumors derived from a review of 11 published clinical studies is presented in Table 1. Response plus stable disease rates for breast, colorectal, and pancreatic cancers are encouraging at 35%, 34% and 36%, respectively. Esophageal cancer, stomach cancer and sarcoma patients benefited the least from treatment with azacitidine, and the response plus stable disease rates reflect this lack of antineoplastic activity. It is worth noting that higher response rates were reported in older studies making comparisons with newer data difficult.

For many of the tumor categories, the response plus stable disease rates are encouraging. For comparison, the response and stable disease rates are provided for treatment of MDS where azacitidine has been proven as an effective drug. The MDS response rate of 23% is only slightly higher and comparable to the response rate in breast cancer at 18%. For further comparison, an objective response rate of 40% in metastatic breast cancer treated with docetaxel has been reported [48]. Also, first-line therapy of metastatic colorectal cancer, the oxaliplatin, infusional fluorouracil and leucovorin (FOLFOX) regimen has been reported as having statistically significant response rates as high as 45% [49]. While not superior to front-line therapy, the response and stable disease rates of our analysis of azacitidine warrant further clinical trials in solid tumors.

We next sought to identify whether or not an optimal clinical dose of azacitidine could be determined in our review of solid tumor clinical trials. Figure 3 shows the response and stable disease rates at three or more dose levels for gastrointestinal cancer, lung cancer, breast cancer and melanoma patients. Included in the charts are the response plus stable disease rate at azacitidine doses for cancer types that had the largest number of patients studied. Our analysis is based on dose per day because total dose information was not reported in some of the studies and precluded making comparisons based on the total dose. We find the highest response and stable disease rate in our analysis occurs not at the highest or maximum tolerated dose, but at a dose of 67.5 mg/m^2/day in all four cancer types examined. The majority of the patients (91 out of 92) treated at this dose were evaluated in the study by Weiss *et al.*, which introduces the possibility of investigator bias, but is nevertheless an important observation that should be considered in designing future clinical trials [50]. The statistical significance of the dose relationship of azacitidine and response rate was calculated in a Fisher's Exact test. The results of this test demonstrates that there is a statistically significant difference ($p < 0.05$) in response at this azacitidine dose of 67.5 mg/m^2/day for gastrointestinal ($p < 0.0001$), lung ($p < 0.0001$) and breast cancer ($p = 0.0089$) patients when compared with other doses, including doses as high as 188 mg/m^2/day. Interestingly, this dose is consistent with more recent data using azacitidine at a nearly identical dose, 75 mg/m^2/day, in hematologic malignancies. The p-value for dose dependence in melanoma patients is less

Table 1. 5-azacytidine activity in solid tumors.

Tumor type	Patient count	Response count	Stable disease count	Progression count	Response rate (%)	Response plus stable disease rate (%)	Dose and schedule	Ref.
Breast	92	17	15	60	18	35	A (n = 29); B (n = 27); C (n = 14); D (n = 8); E (n = 6); F (n = 4); G (n = 3); H (n = 1)	[50,52,83–84]
Female reproductive organs	16	2	0	14	13	13	C (n = 10); H (n = 1); I (n = 4); J (n = 1)	[52,80,81,83]
Gastrointestinal	129	5	26	98	4	24		[50,52,80,81,85–86]
Colorectal	68	3	20	45	4	34	A (n = 26); E (n = 8); H (n = 7); J (n = 1); K (n = 17); L (n = 4); M (n = 2); N (n = 1); O (n = 1); P (n = 1)	[50,52,80,81,83–85]
Esophagus	4	0	0	4	0	0	C (n = 3); H, (n = 1)	[80,81]
Liver	6	0	1	5	0	17	C (n = 4); F (n = 1); H (n=1)	[52,80,81]
Pancreas	11	1	3	7	9	36	C (n = 7); E (n = 2); H (n = 1); M (n = 1)	[80,81,83,84]
Stomach	11	0	0	11	0	0	C (n = 7); E (n = 3); H (n = 1)	[80,81,84]
Miscellaneous gastrointestinal	29	1	2	26	3	10	C (n = 1); R (n = 28)	[80,81,84,86]
Head and neck	23	0	2	21	0	9	C (n = 8); E (n = 14); S (n = 1)	[31,32,80]
Kidney	29	1	5	23	3	21	E (n = 10); K (n = 18); T (n = 1)	[52,80,84,85]
Lung	103	8	14	81	8	21	A (n = 24); E (n = 28); H (n = 5); K (n = 41); M (n = 2); U (n = 1); V (n = 1); W (n = 1)	[50,52,80,81,83–85]
Melanoma	68	3	5	60	4	12	A (n = 12); C (n = 13); E (n = 10); H (n = 1); M (n = 1); P (n = 1); Q (n = 1); T (n = 1); X (n = 26); Y (n = 1); α (n = 1)	[50–52,80,81,83,84]
Sarcoma	26	0	1	25	0	4	C (n = 7); E (n = 3); K (n = 14); P (n = 1); α (n = 1)	[80,83,84,87]
Testicle	4	2	0	2	50	50	C (n = 4)	[80]
Other	83	9	35	39	11	53	A (n = 59); C (n = 15); E (n = 3); H (n = 5); Z, (n = 1)	[50,52,80,81,84]
Solid tumors overall	***573***	***47***	***103***	***423***	***8***	***26***		
Non-Hodgkin's lymphoma	12	2	5	5	17	58	A (n = 8); C (n = 4)	[50,83]
Myelodysplastic syndrome	99	23	37	39	23	61		[15]
	179	51	75	53	28	70		[78]

A: 67.5 mg/m²/day, days 1–10, followed by 101.25 mg/m²/day twice weekly; B: 60 mg/m²/day, days 1–10 then 100 mg/m²/day twice weekly for 8 weeks; C: 150–225 mg/m²/day, days 1–5 every 3 weeks; D: 23–101.25 mg/m²/day, × 10 days; E: 150 mg/m²/day twice weekly for 6 weeks; F: 385–689 mg/m²/total over 10 days; G: 42.1 mg/m²/day × 15 days; H: 50–200 mg/m²/day twice weekly for 8 weeks; I: 275–550 mg/m²/total over 10 days; J: 75.9 mg/m²/day × 10 days; K: 150–200 mg/m²/day 1–5 days every 4 weeks; L: 513–850 mg/m² total over 10 days; M: 84.4 mg/m²/day, × 10 days; N: 92.8 mg/m²/day, × 10 days; O: 42.1 mg/m²/day, × 15 days; P: 50 mg/m²/day, × 10 days; Q: 21.1 mg/m²/day, × 15 days; R: 500–750 mg/m²/total over 10 days; S: 417 mg/m²/total over 10 days; T: 567 mg/m²/total over 10 days; U: 31.6 mg/m²/day, × 11 days; V: 100–150 mg/m²/day twice weekly for 8 weeks; W: 333 mg/m²/total over 10 days; X: 100 mg/m²/day for 10 days every 5 weeks; Y: 23 mg/m²/day, × 10 days; Z: 850 mg/m²/total over 10 days; α: 67.5 mg/m²/day, × 10 days.

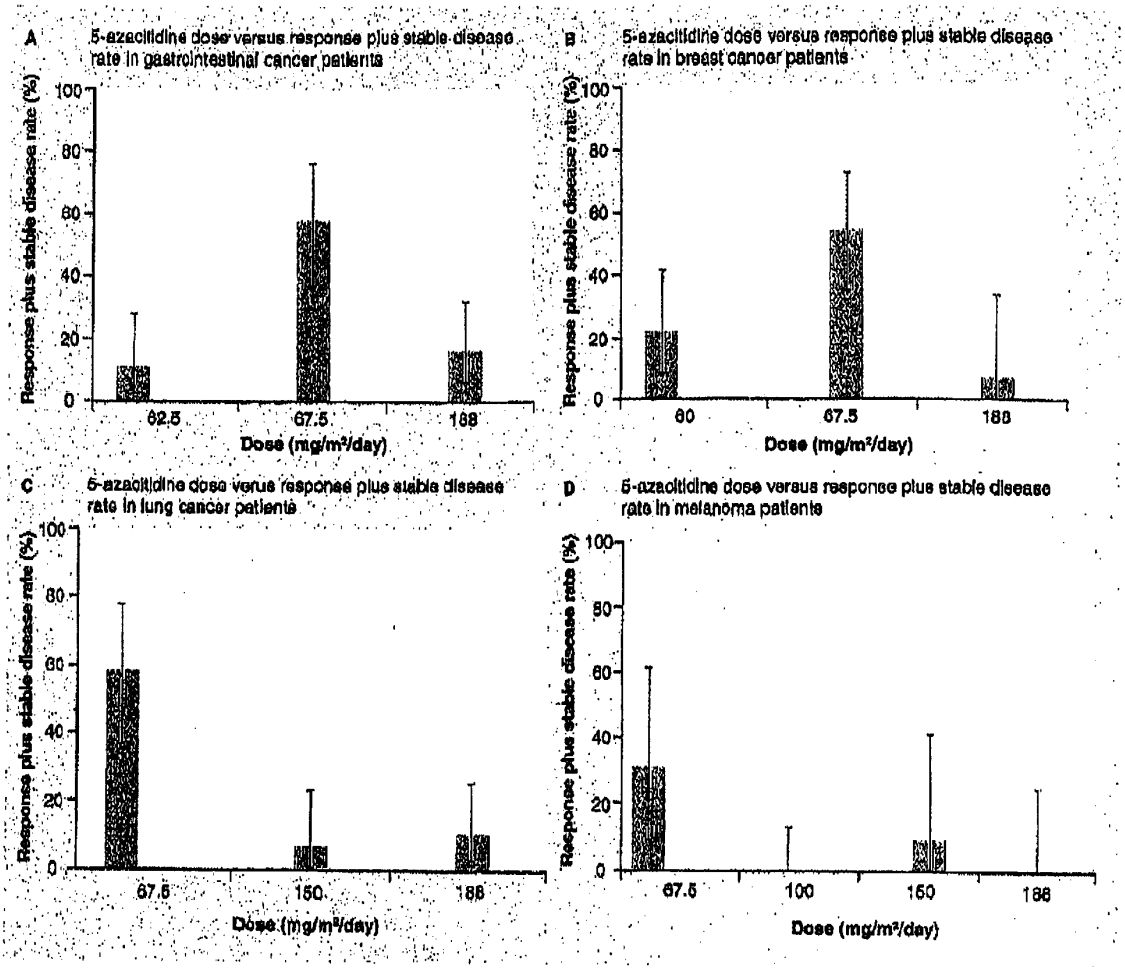


Figure 9. 5-azacytidine dose versus response plus stable disease rate in four different solid tumors. Interestingly, all four solid tumor types have the highest response plus stable disease rate at a 67.5 mg/m²/day dose. Error bars represent the 95% CI. (A) Gastrointestinal cancer, (B) breast cancer, (C) lung cancer and (D) melanoma.

compelling at 0.0380 but does not discredit the notion that 67.5 mg/m²/day may be an optimal dose for melanoma patients. It is important to note that the standard dose of azacitidine for MDS today is 75 mg/m²/day, which is almost the same as the optimal dose in solid tumors.

Dosing schedule data of the original azacitidine studies were captured and categorized into 5-day, 10-day, 15-day and twice-weekly regimens (Table 2). The response plus stable disease rate is maximized at 43% in the 10-day dosing schedule with 273 patients treated. The 5-day response plus stable disease rate was much lower at 13% with 188 patients treated. Limited data for the 15-day treatment schedule looks promising (response plus stable disease rate = 40%); however, only five patients were treated in this manner. Twice-weekly dosing was least successful in our analysis at a response plus stable disease rate of 6% with 112 patients treated. This suggests a 10-day or longer schedule may be optimal, and the current 5- or 7-day schedules of DNA methylation inhibitors for MDS may be suboptimal for solid tumors.

While the majority of trials utilized an intravenous route of azacitidine administration, a couple of studies explored the efficacy of subcutaneously administering azacitidine [51,52]. Table 3 summarizes the overall response plus stable disease rates for subcutaneous and intravenous azacitidine administration. Data from this analysis supports exploring intravenous administration of azacitidine with a response plus stable disease rate of 28% (n = 529),

Table 2. Response and stable disease rates across 5-azacytidine dosing schedules.

Dosing schedule	Patient count	Response count	Stable disease count	Response rate (%)	Stable disease rate (%)	RR + SD (%)
QD × 5	183	6	17	3	9	13
QD × 10	273	32	86	12	32	43
QD × 15	5	2	0	40	0	40
Twice weekly	112	7	0	6	0	6

QD: Every day; RR: Response rate; SD: Stable disease rate.

compared with a substantially lower rate of 4.5% for subcutaneous drug administration (n = 44). This comparison suffers from a low number of patients treated subcutaneously; however, the calculated p-value of 0.0002 attests that the difference in response between routes is statistically significant, and suggests an intravenous route of administration in patients with solid cancers. The antineoplastic activity in solid tumors of decitabine is shown in Table 4. The response plus stable disease rates range from 0–75%; however, this survey suffers from a more limited patient count of 223 patients. The 75% response plus stable disease in thymoma patients is based on four patients, one with an objective response and two with stable disease. While treatment with decitabine resulted in only a couple of responses (one thymoma and one melanoma case), the experience of decitabine in solid tumors is even more limited than azacitidine. However, the response plus stable disease rates with decitabine also support further research on treatment of solid tumors.

An important point to consider is that a stable disease classification can include a substantial increase in tumor volume. Furthermore, this analysis collates a number of studies that inevitably vary in criteria for assigning a stable disease designation. The ability to draw definitive conclusions from this pooled analysis is limited by the heterogeneity of the studies analyzed. Our analysis represents all experience to date with azacitidine and decitabine as single agents in solid tumors and any inferences made from this analysis are intended to guide further research.

In order to fully exploit DNA methylation inhibitors in solid tumors, combination therapy will ultimately be very important. Elegant rational biological combination of DNA methylation inhibitors and HDAC inhibitors are very exciting, but clear clinical synergy has yet to be demonstrated [53,54]. Alternatively, the combination of other interesting biological therapies such as retinoids, immunomodulators and tyrosine kinase inhibitors are all currently under investigation, mostly in hematologic malignancies [55]. A discussion of published combination studies in solid tumors follows and is presented in Table 5.

Combination cytotoxic & hypomethylating agents

The combination of DNA methylation inhibitors and cytotoxic chemotherapy is both scientifically and empirically attractive. Strategies using DNA methylation inhibitors either to open the chromatin for better access by DNA damaging agents or the reversal of epigenetically-induced drug resistance are being tested in cancer [46]. It will be important to be unbiased to the mechanism of DNA methylation inhibitors and not ignore empiric discovery, therefore the pairing of DNA methylation inhibitors with traditional cytotoxic chemotherapy based on clinical experience may also lead to highly effective combination therapy against cancer.

Appleton *et al.* designed a Phase I clinical and pharmacodynamic trial combining decitabine and carboplatin based on work by Plumb in mice indicating that sensitization is schedule dependent and administering decitabine prior to carboplatin achieved the best results [56]. From their clinical trial, they concluded that decitabine combines safely with carboplatin and that the regimen causes epigenetic changes [57]. Out of the ten patients

Table 3. Response and stable disease rate in subcutaneous versus intravenous administration of 5-azacitidine.

Route of administration	Patient count	Response count	Stable disease count	RR + SD (%)
Intravenous	529	45	103	28.0
Subcutaneous	44	2	0	4.5

The Fisher's Exact test yielded a two-tail p-value of 0.0002 suggesting a statistically significant difference between intravenous and subcutaneous response rate plus stable disease rates (RR + SD).
RR: Response rate; SD: Stable disease rate.

Table 4. 5-azacytidine/2′-deoxycytidine activity in solid tumors.

Tumor type	Patient count	Response count	Stable disease count	Progression count	RR + SD (%)	Dose and schedule	Ref.
Breast	4	0	2	2	50	A (n = 4)	[88]
Colorectal	42	0	3	39	7	B (n = 42)	[89]
Female reproductive organs	35	0	2	33	6	B (n = 35)	[90,91]
Head and neck	29	0	5	24	17	A (n = 2); B (n = 27)	[88,89]
Lung	32	0	5	27	16	C (n = 8); D (n = 24)	[92,93]
Melanoma	23	1	5	17	26	A (n = 5); B (n = 18)	[88,89]
Prostate	12	0	2	10	17	B (n = 12)	[94]
Renal	17	0	4	13	24	A (n = 3); B (n = 14)	[88,89]
Testicular	14	0	0	14	0	E (n = 14)	[95]
Thymoma	4	1	2	1	75	A (n = 4)	[88]
Other	11	0	6	5	55	A (n = 8); D (n = 3)	[88]
Solid tumors overall	***223***	***2***	***36***	***185***	***17***		
Myelodysplastic syndrome	89	15	12	62	30	F (n = 89)	[39]
	95	56	13	26	73	G (n = 95)	[76]

A: 2.5–20 mg/m²/day, days 1–5, 8–12 every 4 weeks; B: 75 mg/m² 1 h i.v. 3 x/day every 5 weeks; C: 200–660 mg/m², 8 h i.v., 5–6 weeks apart; D: 60–90 mg/m², 72 h i.v.; E: 60–75 mg/m², 1 h i.v. 3 x/day every 5 weeks; F: 45 mg/m²/day for 3 days (i.v. over 3 h 3 x/day) every 6 weeks; G: 20 mg/m² i.v. daily × 5, 20 mg/ m² subcutaneous daily ×5 or 10 mg/m² i.v. daily × 10.
i.v.: Intravenous; RR: Response rate; SD: Stable disease rate.

treated at the recommended dose, one melanoma patient achieved a partial response that was maintained for eight cycles, and another mesothelioma patient maintained stable disease for six cycles. Two patients treated at a lower decitabine dose of 45 mg/m² (and area under the curve [AUC] 6) also had stable disease. Overall, out of the 30 evaluable patients, the majority of which were heavily pretreated, there was one partial response and three cases of stable disease.

In another Phase I study, Fu combined a dose-escalation (AUC 4 to AUC 5) regimen of carboplatin administered on day 2 with 75 mg/m²/day subcutaneous azacitidine given on days 1–5 in a 28 day cycle in platinum resistant epithelial ovarian cancer patients [58]. Of the three patients evaluable for response, one patient achieved a complete response (by CA125 criteria) and stable disease by imaging criteria, and one patient displayed stable disease. Fu's Phase I study concludes, albeit with a limited number of patients (six patients treated with a total of 16 cycles), that azacitidine 75 mg/m²/day for 5 days in addition to carboplatin AUC 5 on day 2 was well tolerated in heavily pretreated patients.

Pohlmann *et al.* studied the effects of cisplatin and decitabine on patients with advanced squamous cell carcinoma of the cervix in a Phase II trial where 50 mg/m² of decitabine was administered in a 2 h intravenous infusion immediately followed by a 1 h intravenous of 40 mg/m² cisplatin (later reduced to 40 mg/m² due to toxicity) [59]. The 21 evaluable patients had not previously received cytotoxic therapy, but most patients had undergone radiation therapy. Researchers noticed that objective responses were more common in sites not previously irradiated. Of the 21 patients evaluable for tumor response, eight achieved a partial response and five maintained stable disease.

Another Phase I study used a similar schedule of decitabine administered over a 2 h intravenous infusion (45, 67, 90, 120 mg/m², dose escalation) immediately followed by 33 mg/m² cisplatin on three consecutive days, every 21 days, in advanced solid tumors [60]. This study resulted in one partial response in a patient with cervical cancer (response duration 14 weeks), and two minor responses: one in a non-small-cell lung cancer (NSCLC) patient (duration 11 weeks) and another in a cervical cancer patient (duration 13 weeks). The same research group then studied the effects of the recommended dose (from their Phase I trial) of 67 mg/m² decitabine plus 33 mg/m² cisplatin administered using the same dosing schedule to a group of 14 NSCLC patients with disappointing results. Three short minor

Table 3. Summary of combination studies using demethylating agents in treating solid tumors.

Demethylating agent	Cytotoxic/HDAC inhibitor/ immunotherapy therapy	Tumor type(s)	Phase of study (year)	Number of evaluable patients	Response rate (%)	Stable disease rate (%)	Response plus stable disease rate (%)	Ref.
Decitabine: 50 mg/m², d1–3, q21	Cisplatin: 40 mg/m², d1–3, q21 reduced to 30 mg/m² due to toxicity	Cervical	Phase II (2002)	21	38	24	62	[59]
Decitabine: 45, 67, 90, 120 mg/m² dose escalation, 2 h i.v., d1–3, q21	Cisplatin: 33 mg/m², d1–3, q21	NSCLC (n = 8), colorectal (n = 3), renal (n = 3), cervical (n = 3), esophageal (n = 2), pancreas (n = 1), adenocarcinoma of unknown origin (n = 1)	Phase I (2000)	21	14	0	14	[60]
Decitabine: 67 mg/m² 2 h i.v., d1–3, q21	Cisplatin: 33 mg/m², d1–3, q21	NSCLC	Phase II (2000)	14	21	0	21	[60]
Decitabine: 45, 90, 135 mg/m² 6 h i.v., d1, q28	Carboplatin: AUC 5 or AUC 6, d8	Colon (n = 7), breast (n = 5), ovary (n = 5), melanoma (n = 4), sarcoma (n = 4), gallbladder (n = 2), pleural mesothelioma (n = 2), others (n = 6)	Phase I (2007)	30	3	10	13	[57]
Azacitidine: 75 mg/m²/day, d1–5, q28	Carboplatin: AUC 5 i.v., d2	Ovary	Phase I (2006)	3	33	33	67	[58]
Azacitidine: 20, 25, 37.5, 47, 59, 75 and 94 mg/m²/day, SQ for 10 days, q28	Valproic acid: 10–60 mg/kg, PO QD	Colon (n = 11), melanoma of the skin (n = 7), breast (n = 4), head and neck (n = 3), uveal melanoma (n = 3), urothelial (n = 3), thymic (n = 3), thyroid (n = 3), other (n = 18)	Phase I (2008)	55	0	25	25	[62]

AUC: Area under the curve; CIV: Continuous intravenous infusion; HDAC: Histone deacetylase; i.v.: Intravenous; NSCLC: Non-small-cell lung cancer; PO: By mouth; QD: Every day; SQ: Subcutaneous.

Table 3. Summary of combination studies using demethylating agents to treat solid tumors (cont.)

Demethylating agent	Cytotoxic/HDAC inhibitor/ immunotherapy therapy	Tumor type(s)	Phase of study (year)	Number of evaluable patients	Response rate (%)	Stable disease rate (%)	Response plus stable disease rate (%)	Ref.
Azacitidine: 10, 15, 18.75 or 25 mg/m²/day SQ, d1–14, q35 Azacitidine: 75 mg/m²/day SQ, d1–7, q35	Sodium phenylbutyrate: 400 mg/m²/day 24 h CIV, d6 and d13, q35 Sodium phenylbutyrate: 200–400 mg/m²/day CIV, d8–14, q35	Colorectal (n = 7), prostate (n = 5), pancreas, neuroendocrine (n = 1), kidney (n = 3), esophageal (n = 3), breast (n = 2), sarcoma (n = 2), bladder (n = 2), liver (n = 1), lung (n = 1), mesothelia (n = 1)	Phase I (2009)	27	0	4	4	[64]
Azacitidine: 10 or 12.5 mg/m²/day SQ, d1–21, q42	Sodium phenylbutyrate: 400 mg/m²/day 24 h CIV, d6, d13, d20, q42		Interim Phase II (2009)	25	4	8	12	[65]
Azacitidine: 40 mg/m² SQ, d1–6 and 8–10, q28	Entinostat: 7 mg PO d3–10, q28	NSCLC	Phase I (2006)	17	18	29	47	[73]
Decitabine: 0.1, 0.15, 0.2, 0.25 mg/kg dose escalation, SQ daily, d1–5 and d8–12, q12 weeks	IL-2: two cycles of 600,000 IU/kg i.v. q8 × 14 doses, separated by a 2-week break, starting on week 3, q12 weeks	Melanoma (n = 13), ocular melanoma (n = 1), renal cell carcinoma (n = 3)						

AUC: Area under the curve; CIV: Continuous intravenous infusion; HDAC: Histone deacetylase; i.v.: Intravenous; NSCLC: Non-small-cell lung cancer; PO: By mouth; QD: Every day; SQ: Subcutaneous.

responses were documented, which lasted for 4, 16 and 36 weeks with a median patient survival of 15 weeks. The study concluded that the combination of decitabine and cisplatin for treating NSCLC patients was not an improvement over cisplatin alone, which is expected to yield an objective response of approximately 15%. The combination approach also failed to produce 1-year survivors, which falls short of other cisplatin-containing combinations. Combinations of azacitidine and decitabine with traditional chemotherapy clearly has clinical activity, but it is difficult to distinguish the effects of the epigenetic therapy from the cytotoxic therapy.

Histone deacetylase inhibitors & hypomethylating agents

Multiple studies *in vitro* have established the synergy of DNA methylation inhibitors with HDAC inhibitors to activate gene silencing. This has led to a flurry of trials in MDS and solid tumors to combine these two classes of epigenetic therapies [61]. The combination of azacitidine with valproic acid, a HDAC inhibitor, was tested in a Phase I trial on a set of patients with a hetereogeneous mix of advanced cancers. Braiteh *et al.* enrolled 55 patients and followed a treatment regime of 10 consecutive days of subcutaneous azacitidine (20 mg/m^2/day with escalating dose according to a toxicity-based adaptive algorithm) and orally administered valproic acid (10 mg/kg daily titrated by 5 mg/kg every week with a maximum dose of 60 mg/kg/day and a target of a plasma level of 75–100 µg/ml) [62]. A total of 14 patients subjected to the azacitidine/valproic acid combination achieved stable disease with a median duration of 6 months; however, no remissions were reported. Histone acetylation was observed in 61% of study participants, and was noted to be higher in patients achieving stable disease. Evidence of global hypomethylation was found in stable disease patients, but the study could not discern a statistically significant difference in hypomethylation between patients with and without stable disease due to the small size of the study. These results are very encouraging, and the authors suggest further work should concentrate on combining azacitidine with more effective, less toxic HDAC inhibitors such as the combination of azacitidine with MGCD0103, which is currently underway in MDS and AML patients [63].

Another Phase I trial took a combination approach that consisted of subcutaneous azacitidine and a continuous intravenous of sodium phenylbutyrate, a first-generation HDAC inhibitor, to treat a group of 28 patients with dissimilar advanced solid tumors [64]. A total of 27 patients were evaluable for tumor response as one patient demonstrated rapid clinical progression. The study used three dosing schedules that are listed in Table 5. One leiomyosarcoma patient achieved stable disease for 4.5 months and received four cycles of 75 mg/m^2/day subcutaneous azacitidine days 1–7 and 200 mg/m^2/day sodium phenylbutenate continuous intravenous on days 8–14, every 35 days. Overall, Lin *et al.* concluded that their trial failed to demonstrate any real evidence for clinical benefit [64].

Juergens *et al.* also combined azacitidine with a HDAC inhibitor, entinostat, in a Phase II trial in relapsed advanced lung cancer. In the interim analysis, 25 patients have been recruited with histologically confirmed recurrent NSCLC. Patients were administered 40 mg/m^2 of azacitidine subcutaneously on days 1–5 and 8–10, and 7 mg entinostat orally on days 3 and 10 of a 28 day cycle. One patient had a complete response and another two had stabilization of disease. One patient has stable disease for 4 months and the other patient continues to have stable disease after 16 months [65].

The combination of a HDAC inhibitor and a DNA methylation inhibitor is attractive from a biological rationale, but the data to date in solid tumors is too nascent to demonstrate a clear synergy especially without a randomized clinical trial. Clearly, there is more experience with this epigenetic combination in hematologic malignancies [53,66], but even this data is not conclusive and one should be cautious in extrapolating success or failures in MDS and leukemia to solid tumors.

Immunotherapy & hypomethylating agents

DNA methyltransferase inhibitors are known to reactivate tumor antigens and this has created interest in combining DNA methylation inhibitors with immunotherapies [67–70]. Melanoma and renal cell cancer cell lines have demonstrated downregulation of MHC class I expression and tumor antigen expression [68,71–73]. Furthermore, this downregulation of apoptosis and antigen presentation has been linked to repression of gene transcription due to hypermethylation of CpG islands in regulatory regions of genes responsible for apoptosis and antigen presentation. Tumors that are resistant to high-dose IL-2, therefore, may regain sensitivity after treatment with hypomethylating

agents. The Phase I trial carried out by Gollob *et al.* in patients with melanoma or renal cell carcinoma sought to gain evidence to support this theory and utilized a 12 week cycle in which subcutaneous decitabine (0.1–0.25 mg/kg, dose escalation) was administered on days 1–5 and 8–12 followed by immunotherapy (two cycles of 600,000 IU/kg IL-2 intravenous q8 h × 14 doses separated by a 2-week break, starting on week 3) [71]. The trial evaluated 17 out of the 21 enrolled patients for response including 13 melanoma, one ocular melanoma and three renal cell carcinoma patients. In melanoma patients, the study yielded three major responses and two tumor regressions (one ocular melanoma patient and one melanoma patient), which were not sufficient to categorize as partial by the Response Evaluation Criteria in Solid Tumors. In TABLE 5, we count these tumor regressions as stable disease. In addition, three renal cell cancer patients achieved stable disease. The major success of the trial by Gollob *et al.* favors the combination of decitabine and IL-2 for melanoma and renal cell cancer patients.

Conclusion

We began this study in order to determine if the DNA methylation inhibitors, azacitidine and decitabine, had activity in solid tumors. The answer is clearly yes. With a careful review of previously published work we have found that DNA methylation inhibitors can have response rates as high as 18% in breast cancer. We found variable but clear activity of azacitidine and decitabine against many different cancers. The total number of patients treated was small and therefore our experience with DNA methylation inhibitors is limited. In addition, as these were investigational studies, the patients treated were heavily pretreated with other cytotoxic chemotherapy agents before being treated with a DNA methylation inhibitor making the clinical activity observed all the more impressive.

Differences in dosing-schedule responses yielded interesting results in favor of using a daily, azacitidine dosing regimen for 10 days over other schedules, which supports an earlier study using decitabine by Issa *et al.* and *in vitro* results favoring prolonged exposures [74,75]. In addition our data suggest an intravenous route of administration may be favorable compared with subcutaneous administration.

Unfortunately, most of the clinical trials reviewed were conducted prior to the knowledge that azacitidine and decitabine inhibited DNA methylation and therefore the optimal dose and schedule of DNA methylation inhibitors has yet to be fully studied in patients with solid tumors. Laboratory studies have demonstrated that azacitidine and decitabine optimally inhibit DNA methylation as epigenetic therapies when used at lower doses with prolonged exposures [17,74,76,77]. This observation in cell culture is consistent with what is observed clinically in patients with MDS who are treated with azacitidine and decitabine. Clearly, the drugs are more active well below their maximum tolerated dose and for prolonged exposures. The impact of using azacitidine and decitabine at the optimal epigenetic dose instead of the optimal cytotoxic dose is yet unknown, however, one would expect a significant increase in anticancer activity [76,77].

Clinical studies have traditionally used surrogate markers for survival such as response rate or time to tumor progression. The use of these surrogate markers is for convenience and allows the efficacy of a drug to be determined more rapidly. Recent studies of azacitidine in MDS cast doubt on the utility of response rate as a surrogate for overall survival. Intensive chemotherapy has a higher response rate in MDS when compared with azacitidine, but this does not translate into a survival benefit [78]. In addition, partial responses, hematologic improvement and even stable disease was associated with a trend to increased survival [37]. This poses the possibility that epigenetic agents may increase survival and benefit patients with cancer without decreasing tumor size, and adds new challenges to the design of cancer trials using epigenetic agents.

Although it is difficult to compare the activity of decitabine to azacitidine in solid tumors it appears there may be a trend in favor of azacitidine in solid tumors. Partial responses are reported for azacitidine in solid tumors but fewer responses are reported for decitabine in solid tumors. This may suggest that mechanisms other than DNA methylation inhibition play a role in the anticancer activity of azacitidine such as incorporation into RNA [79], but to date, no clear data exists to support this hypothesis. Alternatively, the trials using decitabine were done at a later date and more standardized response criteria could have been used. There are no clinical trials comparing the efficacy of azacitidine and decitabine in solid tumors, and studies comparing azacitidine directly to decitabine in MDS or leukemia may be difficult to extrapolate to other cancers.

Combinations of azacitidine and decitabine based on sound scientific rationale or empiric experience are another path forward for these epigenetic therapies. Clearly, combinations of DNA methyltransferase inhibitors with HDAC inhibitors, immunotherapies and cytotoxic chemotherapy have demonstrated early clinical promise. However, it will be difficult to distinguish the effect of the DNA methylation inhibitor from the other agent that is given in combination. Furthermore, there should be balance in rational versus empiric combinations.

One difficulty in treating solid tumors compared with hematologic malignancies is the limited number of dividing cells in solid cancers. Azacitidine and decitabine are S-phase specific drugs and need to be incorporated into the DNA of dividing cells in order to carry out their epigenetic effect. Unfortunately, the azacytosine ring of azacitidine and decitabine are exquisitely labile in aqueous solution and even more sensitive to cytidine deaminase which is abundant in the serum. Therefore, next-generation methylation inhibitors such as zebularine, S-110 and NPEOC-DAC, which are more stable, may offer increased efficacy in patients with all cancer types [24,25].

Future perspective

The field of epigenetics and epigenetic therapy has only just begun. Improved understanding of DNA methylation patterns and the overall structure of the epigenome will lead to new treatments not only for cancer but also other diseases. DNA methylation inhibitors will move quickly from the treatment of hematologic malignancies to other cancers, and DNA methylation inhibitors represent only the beginning for an entire new class of drugs based on the field of epigenetics.

Financial & competing interests disclosure

Allen S Yang is currently a full-time employee of Amgen, Inc. and those current duties are not related to this work. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Executive summary

- The DNA methylation inhibitors, azacitidine (Vidaza®) and decitabine (Dacogen®), are old drugs that had been used in clinical trials as early as the 1970s.
- DNA methylation inhibitors are S-phase specific drugs that are optimal at low doses and prolonged schedules in order to fully exploit their ability to inhibit DNA methylation.
- The use of azacitidine and decitabine in solid tumors was completed prior to the knowledge of the drugs' ability to inhibit DNA methylation and therefore the optimal doses and schedules were not used.

Methods & data collection

- We reviewed 11 clinical trials that used azacitidine in patients with solid tumors (694 patients total).
- We reviewed eight clinical trials that used decitabine in patients with solid tumors (256 patients total).
- DNA methylation inhibitors have been previously tested in a variety of solid organ cancers, including breast, colorectal, prostate, pancreas, lung, head and neck, melanoma, renal, testicular, female reproductive organsand so on.

Results

- Azacitidine and decitabine have clear clinical activity in solid tumors with response rates as high as 50% depending on the tumor type.
- Azacitidine appears to have an optimal biological dose in solid tumors, and may be more effective at lower doses and for prolonged exposures.
- Azacitidine may be more effective via intravenous route of administration than subcutaneous.
- Combinations of DNA methyltransferase inhibitors have been tested with cytotoxic chemotherapy, histone deacetylase inhibitors and immunotherapy.

Conclusion

- There is ample clinical evidence that azacitidine and decitabine have activity in solid tumors.
- Previous studies with azacitidine and decitabine are likely to have used suboptimal doses and schedules.
- Additional studies with azacitidine and decitabine using lower doses with prolonged exposures to exploit the potential 'epigenetic effects' are warranted.
- Combination therapy strategies as well as next-generation DNA methylation inhibitors are in development.

Bibliography

Papers of special note have been highlighted as:
* of interest
** of considerable interest

1 Esteller M: Epigenetics in cancer. *N. Engl. J. Med.* 358(11), 1148–1159 (2008).

2 Herman JG, Baylin SB: Gene silencing in cancer in association with promoter hypermethylation. *N. Engl. J. Med.* 349(21), 2042–2054 (2003).

3 Yoo CB, Jones PA: Epigenetic therapy of cancer: past, present and future. *Nat. Rev. Drug Discov.* 5(1), 37–50 (2006).

4 Gilbert J, Gore SD, Herman JG, Carducci MA: The clinical application of targeting cancer through histone acetylation and hypomethylation. *Clin. Cancer Res.* 10(14), 4589–4596 (2004).

5 Bollati V, Baccarelli A, Hou L *et al.*: Changes in DNA methylation patterns in subjects exposed to low-dose benzene. *Cancer Res.* 67(3), 876–880 (2007).

6 Jirtle RL, Skinner MK: Environmental epigenomics and disease susceptibility. *Nat. Rev. Genet.* 8(4), 253–262 (2007).

7 Jones PA, Baylin SB: The fundamental role of epigenetic events in cancer. *Nat. Rev. Genet.* 3(6), 415–428 (2002).

** Good review of epigenetics and cancer for those new to the field.

8 Laird PW: Cancer epigenetics. *Hum. Mol. Genet.* 14(Spec. No. 1), R65–R76 (2005).

9 Garcia-Manero G, Jelu S, Daniel J *et al.*: Aberrant DNA methylation in pediatric patients with acute lymphocytic leukemia. *Cancer* 97(3), 695–702 (2003).

10 Melki JR, Warnecke P, Vincent PC, Clark SJ: Increased DNA methyltransferase expression in leukaemia. *Leukemia* 12(3), 311–316 (1998).

11 Roman-Gomez J, Castillejo JA, Jimenez A, Barrios M, Heiniger A, Torres A: The role of DNA hypermethylation in the pathogenesis and prognosis of acute lymphoblastic leukemia. *Leuk. Lymphoma* 44(11), 1855–1864 (2003).

12 Toyota M, Kopecky KJ, Toyota MO, Jair KW, Willman CL, Issa JP: Methylation profiling in acute myeloid leukemia. *Blood* 97(9), 2823–2829 (2001).

13 Christmann M, Pick M, Lage H, Schadendorf D, Kaina B: Acquired resistance of melanoma cells to the antineoplastic agent fotemustine is caused by reactivation of the DNA repair gene *MGMT*. *Int. J. Cancer* 92(1), 123–129 (2001).

14 Esteller M, Garcia-Foncillas J, Andion E *et al.*: Inactivation of the DNA-repair gene *MGMT* and the clinical response of gliomas to alkylating agents. *N. Engl. J. Med.* 343(19), 1350–1354 (2000).

* Important link between a DNA methylation biomarker and clinical phenotype.

15 Glover AB, Leyland-Jones BR, Chun HG, Davies B, Hoth DF: Azacitidine: 10 years later. *Cancer Treat. Rep.* 71(7–8), 737–746 (1987).

* Good overview of the clinical experience with azacitidine prior to the knowledge of its epigenetic effects.

16 Christman JK: 5-azacytidine and 5-aza-2′-deoxycytidine as inhibitors of DNA methylation: mechanistic studies and their implications for cancer therapy. *Oncogene* 21(35), 5483–5495 (2002).

17 Jones PA, Taylor SM: Cellular differentiation, cytidine analogs and DNA methylation. *Cell* 20(1), 85–93 (1980).

** Classic paper in the field that first describes the epigenetic effects of azacitidine and decitabine.

18 Taylor SM, Constantinides PA, Jones PA: 5-azacytidine, DNA methylation, and differentiation. *Curr. Top. Microbiol. Immunol.* 108, 115–127 (1984).

19 Silverman LR, Demakos EP, Peterson BL *et al.*: Randomized controlled trial of azacitidine in patients with the myelodysplastic syndrome: a study of the cancer and leukemia group b. *J. Clin. Oncol.* 20(10), 2429–2440 (2002).

* First randomized clinical trial of azacitidine demonstrating a clinical benefit in myelodysplastic syndrome.

20 Choi SH, Byun HM, Kwan JM, Issa JP, Yang AS: Hydroxycarbamide in combination with azacitidine or decitabine is antagonistic on DNA methylation inhibition. *Br. J. Haematol.* 138(5), 616–623 (2007).

21 Antonsson BE, Avramis VI, Nyce J, Holcenberg JS: Effect of 5-azacytidine and congeners on DNA methylation and expression of deoxycytidine kinase in the human lymphoid cell lines CCRF/CEM/0 AND CCRF/CEM/DCK-1. *Cancer Res.* 47(14), 3672–3678 (1987).

22 Cheng JC, Matsen CB, Gonzales FA *et al.*: Inhibition of DNA methylation and reactivation of silenced genes by zebularine. *J. Natl Cancer Inst.* 95(5), 399–409 (2003).

23 Randerath K, Tseng WC, Harris JS, Lu LJ: Specific effects of 5-fluoropyrimidines and 5-azapyrimidines on modification of the 5 position of pyrimidines, in particular the synthesis of 5-methyluracil and 5-methylcytosine in nucleic acids. *Recent Results Cancer Res.* 84, 289–297 (1983).

24 Yoo CB, Jeong S, Egger G *et al.*: Delivery of 5-aza-2′-deoxycytidine to cells using oligodeoxynucleotides. *Cancer Res.* 67(13), 6400–6408 (2007).

** Evaluation of potential prodrugs of decitabine, including SGI-110.

25 Byun H-M, Choi S-H, Laird PW, Trinh B, Marquez VE, Yang AS: 2′-deoxy-n4-[2-(4-nitrophenyl) ethoxycarbonyl]-5-azacytidine a novel inhibitor of DNA methyltransferase that requires activation by human carboxylesterase 1. *Cancer Lett.* 266(2), 238–248 (2008).

26 Wyczechowska D, Fabianowska-Majewska K: The effects of cladribine and fludarabine on DNA methylation in k562 cells. *Biochem. Pharmacol.* 65(2), 219–225 (2003).

27 Lin X, Asgari K, Putzi MJ *et al.*: Reversal of GSTP1 CpG island hypermethylation and reactivation of π-class glutathione S-transferase (GSTP1) expression in human prostate cancer cells by treatment with procainamide. *Cancer Res.* 61(24), 8611–8616 (2001).

28 Parker BS, Cutts SM, Nudelman A *et al.*: Mitoxantrone mediates demethylation and reexpression of cyclin D2, estrogen receptor and 14.3.3σ in breast cancer cells. *Cancer Biol. Ther.* 2(3), 259–263 (2003).

29 Richardson B, Cornacchia E, Golbus J, Marbaum J, Strahler J, Hanash S: *N*-acetylprocainamide is a less potent inducer of T cell autoreactivity than procainamide. *Arthritis Rheum.* 31(8), 995–999 (1988).

30 Thomas TJ, Messner RP: Effects of lupus-inducing drugs on the B to Z transition of synthetic DNA. *Arthritis Rheum.* 29(5), 638–645 (1986).

31 Villar-Garea A, Fraga MF, Espada J, Esteller M: Procaine is a DNA-demethylating agent with growth-inhibitory effects in human cancer cells. *Cancer Res.* 63(16), 4984–4989 (2003).

32 Zacharias W, Koopman WJ: Lupus-inducing drugs alter the structure of supercoiled circular DNA domains. *Arthritis Rheum.* 33(3), 366–374 (1990).

33 Chuang JC, Yoo CB, Kwan JM *et al.*: Comparison of biological effects of non-nucleoside DNA methylation inhibitors versus 5-aza-2′-deoxycytidine. *Mol. Cancer Ther.* 4(10), 1515–1520 (2005).

* Comparison of azacitidine and decitabine versus other DNA methylation inhibitors.

34 Davis AJ, Gelmon KA, Siu LL *et al.*: Phase I and pharmacologic study of the human DNA methyltransferase antisense oligodeoxynucleotide MG98 given as a 21-day continuous infusion every 4 weeks. *Invest. New Drugs* 21(1), 85–97 (2003).

35 Fang MZ, Wang Y, Ai N *et al.*: Tea polyphenol (-)-epigallocatechin-3-gallate inhibits DNA methyltransferase and reactivates methylation-silenced genes in cancer cell lines. *Cancer Res.* 63(22), 7563–7570 (2003).

36 Shi ST, Wang ZY, Smith TJ *et al.*: Effects of green tea and black tea on 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone bioactivation, DNA methylation, and lung tumorigenesis in A/J mice. *Cancer Res.* 54(17), 4641–4647 (1994).

37 List AF, Fenaux GJ, Hellstrom-Lindber E *et al.*: Effect of azacitidine (aza) on overall survival in higher-risk myelodysplastic syndromes (MDS) without complete remission. *J. Clin. Oncol.* 26(15S), 7006 (2008).

38 Wijermans P, Suciu S, Baila L *et al.*: Low dose decitabine versus best supportive care in elderly patients with intermediate or high risk MDS not eligible for intensive chemotherapy: final results of the randomized Phase III study (06011) of the EORTC leukemia and German MDS study groups. *Blood* 112, 226 (2008) (ASH Annual Meeting Abstracts).

39 Kantarjian H IJ, Rosenfeld CS, Bennett JM *et al.*: Decitabine improves patient outcomes in myelodysplastic syndromes: results of a Phase III randomized study. *Cancer* 106(8), 1794–1803 (2006).

40 Wijermans P, Lubbert M, Verhoef G *et al.*: Low-dose 5-aza-2′-deoxycytidine, a DNA hypomethylating agent, for the treatment of high-risk myelodysplastic syndrome: a multicenter Phase II study in elderly patients. *J. Clin. Oncol.* 18(5), 956–962 (2000).

41 Esteller M: DNA methylation and cancer therapy: new developments and expectations. *Curr. Opin. Oncol.* 17(1), 55–60 (2005).

42 Garcia-Manero G, Gore SD: Future directions for the use of hypomethylating agents. *Semin. Hematol.* 42(3 Suppl. 2), S50–S59 (2005).

43 Graham JS, Kaye SB, Brown R: The promises and pitfalls of epigenetic therapies in solid tumours. *Eur. J. Cancer* 45(7), 1129–1136 (2009).

44 Issa JP, Kantarjian HM: Targeting DNA methylation. *Clin. Cancer Res.* 15(12), 3938–3946 (2009).

45 Plimack ER, Stewart DJ, Issa JP: Combining epigenetic and cytotoxic therapy in the treatment of solid tumors. *J. Clin. Oncol.* 25(29), 4519–4521 (2007).

46 Sigalotti L, Coral S, Fratta E *et al.*: Epigenetic modulation of solid tumors as a novel approach for cancer immunotherapy. *Semin. Oncol.* 32(5), 473–478 (2005).

47 van Groeningen CJ, Leyva A, O'Brien AM, Gall HE, Pinedo HM: Phase I and pharmacokinetic study of 5-aza-2′-deoxycytidine (NSC 127716) in cancer patients. *Cancer Res.* 46(9), 4831–4836 (1986).

48 Katsumata N, Watanabe T, Minami H *et al.*: Phase III trial of doxorubicin plus cyclophosphamide (ac), docetaxel, and alternating ac and docetaxel as front-line chemotherapy for metastatic breast cancer: Japan clinical oncology group trial (JCOG9802). *Ann. Oncol.* 20(7), 1210–1215 (2009).

49 Meyerhardt JA, Mayer RJ: Systemic therapy for colorectal cancer. *N. Engl. J. Med.* 352(5), 476–487 (2005).

50 Weiss AJ, Metter Ge, Neulon TF *et al.*: Phase II study of 5-azacytidine in solid tumors. *Cancer Treat. Rep.* 61(1), 55–58 (1977).

51 Bellet RE, Catalano RB, Mastrangelo MJ, Berd D: Phase II study of subcutaneously administered 5-azacytidine (NSC-102816) in patients with metastatic malignant melanoma. *Med. Pediatr. Oncol.* 4(1), 11–15 (1978).

52 Bellet RE, Mastrangelo MJ, Engstrom PB, Strawitz JG, Weiss AJ, Yarbro JW: Clinical trial with subcutaneously administered 5-azacytidine (NSC-102816). *Cancer Chemother. Rep.* 58(2), 217–222 (1974).

53 Garcia-Manero G, Kantarjian HM, Sanchez-Gonzalez B *et al.*: Phase 1/2 study of the combination of 5-aza-2′-deoxycytidine with valproic acid in patients with leukemia. *Blood* 108(10), 3271–3279 (2006).

54 Gore SD: Combination therapy with DNA methyltransferase inhibitors in hematologic malignancies. *Nat. Clin. Pract. Oncol.* 2(Suppl. 1), S30–S35 (2005).

55 Oki Y, Kantarjian HM, Gharibyan V *et al.*: Phase II study of low-dose decitabine in combination with imatinib mesylate in patients with accelerated or myeloid blastic phase of chronic myelogenous leukemia. *Cancer* 109(5), 899–906 (2007).

56 Plumb JA, Strathdee G, Sludden J, Kaye SB, Brown R: Reversal of drug resistance in human tumor xenografts by 2′-deoxy-5-azacytidine-induced demethylation of the *HMLH1* gene promoter. *Cancer Res.* 60(21), 6039–6044 (2000).

57 Appleton K, Mackay HJ, Judson I *et al.*: Phase I and pharmacodynamic trial of the DNA methyltransferase inhibitor decitabine and carboplatin in solid tumors. *J. Clin. Oncol.* 25(29), 4603–4609 (2007).

58 Fu S: A Phase I study of a hypomethylating agent azacitidine in combination with carboplatin in patients with platinum resistant epithelial ovarian cancer. *J. Clin. Oncol.* 24, 5087 (2006) (Meeting Abstracts).

59 Pohlmann P, Dilcone LP, Cancella Al *et al.*: Phase II trial of cisplatin plus decitabine, a new DNA hypomethylating agent, in patients with advanced squamous cell carcinoma of the cervix. *Am. J. Clin. Oncol.* 25(5), 496–501 (2002).

60 Schwarzmann G, Schumermann H, Gorlat CN *et al.*: A Phase I trial of cisplatin plus decitabine, a new DNA-hypomethylating agent, in patients with advanced solid tumors and a follow-up early Phase II evaluation in patients with inoperable non-small cell lung cancer. *Invest. New Drugs* 18(1), 85–91 (2000).

61 Fandy TE, Carraway H, Gore SD: DNA demethylating agents and histone deacetylase inhibitors in hematologic malignancies. *Cancer J.* 13(1), 40–48 (2007).

62 Braiteh F, Soriano AO, Garcia-Manero G *et al.*: Phase I study of epigenetic modulation with 5-azacytidine and valproic acid in patients with advanced cancers. *Clin. Cancer Res.* 14(19), 6296–6301 (2008).

63 Garcia-Manero G, Yang AS, Giles F *et al.*: Phase I/II study of the oral isotype-selective histone deacetylase (HDAC) inhibitor MGCD0103 in combination with azacitidine in patients (pts) with high-risk myelodysplastic syndrome (MDS) or acute myelogenous leukemia (AML). *Blood* 108 (2006) (ASH Annual Meeting Abstract No. 1954).

64 Lin J, Gilbert J, Rudek MA *et al.*: A Phase I dose-finding study of 5-azacytidine in combination with sodium phenylbutyrate in patients with refractory solid tumors. *Clin. Cancer Res.* 15(19), 6241–6249 (2009).

65 Juergens RA, Vendetti F, Coleman B *et al.*: Interim analysis of a Phase II trial of 5-azacitidine (5AC) and entinostat (SNDX-275) in relapsed advanced lung cancer (NSCLC). *J. Clin. Oncol.* 27, 8055 (2009) (Meeting abstracts).

66 Fandy TE, Herman JG, Kerns P *et al.*: Early epigenetic changes and DNA damage do not predict clinical response in an overlapping schedule of 5-azacytidine and entinostat in patients with myeloid malignancies. *Blood* 114(13), 2764–2773 (2009).

▪ Detailed translational study combining azacitidine with entinostat.

67 Aparicio A, North B, Barske L *et al.*: Line-1 methylation in plasma DNA as a biomarker of activity of DNA methylation inhibitors in patients with solid tumors. *Epigenetics* 4(3), 176–184 (2009).

68 Coral S, Sigalotti L, Altomonte M *et al.*: 5-aza-2′-deoxycytidine-induced expression of functional cancer testis antigens in human renal cell carcinomas: immunotherapeutic implications. *Clin. Cancer Res.* 8(8), 2690–2695 (2002).

69 De Smet C, De Backer O, Faraoni I, Lurquin C, Brasseur F, Boon T: The activation of human gene *MAGE-1* in tumor cells is correlated with genome-wide demethylation. *Proc. Natl Acad. Sci. USA* 93(14), 7149–7153 (1996).

70 Karpf AR, Lasek AW, Ririe TO, Hanks AN, Grossman D, Jones DA: Limited gene activation in tumor and normal epithelial cells treated with the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine. *Mol. Pharmacol.* 65(1), 18–27 (2004).

71 Gollob JA, Schmbi CJ, Peterson BL *et al.*: Phase I trial of sequential low-dose 5-aza-2′-deoxycytidine plus high-dose intravenous bolus interleukin-2 in patients with melanoma or renal cell carcinoma. *Clin. Cancer Res.* 12(15), 4619–4627 (2006).

72 Weiser TS, Guo ZS, Ohnmacht GA *et al.*: Sequential 5-aza-2′-deoxycytidine-depsipeptide FR901228 treatment induces apoptosis preferentially in cancer cells and facilitates their recognition by cytolytic T lymphocytes specific for NY-ESO-1. *J. Immunother. (1991)* 24(2), 151–161 (2001).

73 Serrano A, Tanzarella S, Lionello I *et al.*: Rexpression of HLA class I antigens and restoration of antigen-specific CTL response in melanoma cells following 5-aza-2′-deoxycytidine treatment. *Int. J. Cancer* 94(2), 243–251 (2001).

74 Issa JP, Garcia-Manero G, Giles FJ *et al.*: Phase 1 study of low-dose prolonged exposure schedules of the hypomethylating agent 5-aza-2′-deoxycytidine (decitabine) in hematopoietic malignancies. *Blood* 103(5), 1635–1640 (2004).

▪ Clinical trial trying to define the optimal biological dose of decitabine.

75 Qin T, Youssef EM, Jelinek J *et al.*: Effect of cytarabine and decitabine in combination in human leukemic cell lines. *Clin. Cancer Res.* 13(14), 4225–4232 (2007).

76 Kantarjian H, Oki Y, Garcia-Manero G *et al.*: Results of a randomized study of 3 schedules of low-dose decitabine in higher-risk myelodysplastic syndrome and chronic myelomonocytic leukemia. *Blood* 109(1), 52–57 (2007).

77 Yang AS, Doshi KD, Choi SW *et al.*: DNA methylation changes after 5-aza-2′-deoxycytidine therapy in patients with leukemia. *Cancer Res.* 66(10), 5495–5503 (2006).

▪ Translational study looking at the DNA methylation changes induced by decitabine.

78 Fenaux P, Mufti GJ, Hellstrom-Lindberg E *et al.*: Efficacy of azacitidine compared with that of conventional care regimens in the treatment of higher-risk myelodysplastic syndromes: a randomised, open-label, Phase III study. *Lancet Oncol.* 10(3), 223–232 (2009).

▪▪ First randomized trial to demonstrate a clear survival benefit for azacitidine-treated patients.

79 Schaefer M, Hagemann S, Hanna K, Lyko F: Azacytidine inhibits RNA methylation at DNMT2 target sites in human cancer cell lines. *Cancer Res.* 69(20), 8127–8132 (2009).

▪ Potentially important observation that azacitidine can affect RNA methylation.

80 Quagliana JM, O'Bryan RM, Baker L *et al.*: Phase II study of 5-azacytidine in solid tumors. *Cancer Treat. Rep.* 61(1), 51–54 (1977).

81 Vogler WR, Arkun S, Velez-Garcia E: Phase I study of twice weekly 5-azacytidine (NSC-102816). *Cancer Chemother. Rep.* 58(6), 895–899 (1974).

82 Cunningham TJ, Nemoto T, Rosner D *et al.*: Comparison of 5-azacytidine (NSC-102816) with CCNU (NSC-79037) in the treatment of patients with breast cancer and evaluation of the subsequent use of cyclophosphamide (NSC-26271). *Cancer Chemother. Rep.* 58(5 Pt 1), 677–681 (1974).

83 Weiss AJ, Stambaugh JE, Mastrangelo MJ, Laucius JF, Bellet RE: Phase I study of 5-azacytidine (NSC-102816). *Cancer Chemother. Rep.* 56(3), 413–419 (1972).

84 Velez-Garcia E, Vogler WR, Bartolucci AA, Arkun SN: Twice weekly 5-azacytidine infusion in disseminated metastatic cancer: a Phase II study. *Cancer Treat. Rep.* 61(9), 1675–1677 (1977).

85 Lomen PL, Baker LH, Nell GL, Samson MK: Phase I study of 5-azacytidine (NSC-102816) using 24-hour continuous infusion for 5 days. *Cancer Chemother. Rep.* 59(6), 1123–1126 (1975).

86 Moertel CG, Schutt AJ, Reitemeier RJ, Hahn RG: Phase II study of 5-azacytidine (NSC-102816) in the treatment of advanced gastrointestinal cancer. *Cancer Chemother. Rep.* 56(5), 649–652 (1972).

87 Srinivasan U, Reaman GH, Poplack DG, Glaubiger DL, Levine AS: Phase II study of 5-azacytidine in sarcomas of bone. *Am. J. Clin. Oncol.* 5(4), 411–415 (1982).

88 Stewart DJ, Issa JP, Kurzrock R *et al.*: Decitabine effect on tumor global DNA methylation and other parameters in a Phase I trial in refractory solid tumors and lymphomas. *Clin. Cancer Res.* 15(11), 3881–3888 (2009).

▪ More recent trial of decitabine in patients with solid tumors.

89 Abele R, Clavel M, Dodion P *et al.*: The EORTC early clinical trials cooperative group experience with 5-aza-2′-deoxycytidine (NSC 127716) in patients with colo-rectal, head and neck, renal carcinomas and malignant melanomas. *Eur. J. Cancer Clin. Oncol.* 23(12), 1921–1924 (1987).

90 Sessa C, Ten Bokkel Huinink W, Scoccr G, Renard J, Cavalli F: Phase II study of 5-aza-2′-deoxycytidine in advanced ovarian carcinoma. The EORTC early clinical trials group. *Eur. J. Cancer* 26(2), 137–138 (1990).

91 Vermorken JB, Tumolo S, Rozencdaul KJ, Gunstalla JP, Splinter TA, Renard J: 5-aza-2′-deoxycytidine in advanced or recurrent cancer of the uterine cervix. *Eur. J. Cancer* 27(2), 216–217 (1991).

92 Schrump DS, Fischette MR, Nguyen DM *et al.*: Phase I study of decitabine-mediated gene expression in patients with cancers involving the lungs, esophagus, or pleura. *Clin. Cancer Res.* 12(19), 5777–5785 (2006).

▪ Recent translational study of decitabine in patients with thoracic cancers.

93 Momparler RL, Bouffard DY, Momparler LF, Dionne J, Belanger K, Ayoub J: Pilot Phase I–II study on 5-aza-2′-deoxycytidine (decitabine) in patients with metastatic lung cancer. *Anticancer Drugs* 8(4), 358–368 (1997).

94 Thibault A, Figg WD, Bergan RC *et al.*: A Phase II study of 5-aza-2′deoxycytidine (decitabine) in hormone independent metastatic (D2) prostate cancer. *Tumori* 84(1), 87–89 (1998).

95 Clavel M, Monfardini S, Possa S, Smyth J, Renard J, Kaye SB: 5-aza-2′-deoxycytidine (NSC 127716) in non-seminomatous testicular cancer. Phase II from the EORTC early clinical trials cooperative group and genito-urinary group. *Ann. Oncol.* 3(5), 399–400 (1992).

BIOCHEMICAL PHARMACOLOGY 74 (2007) 659–671

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Commentary

HDAC inhibitors: Clinical update and mechanism-based potential

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ARTICLE INFO

Article history:
Received 5 March 2007
Accepted 4 April 2007

Keywords:
Histone deacetylase inhibitors
Chromatin modification
Epigenetic regulation
Vorinostat
Belinostat
Romidepsin
LBH589
MGCD0103
MS-275

ABSTRACT

Recently, the role of transcriptional repression through epigenetic modulation in carcinogenesis has been clinically validated with several inhibitors of histone deacetylases and DNA methyltransferases. It has long been recognized that epigenetic alterations of tumor suppressor genes was one of the contributing factors in carcinogenesis. Inhibitors of histone deacetylase (HDAC) de-repress genes that subsequently result in growth inhibition, differentiation and apoptosis of cancer cells. Vorinostat (SAHA), romidepsin (depsipeptide, FK-228), belinostat (PXD101) and LAQ824/LBH589 have demonstrated therapeutic benefit as monotherapy in cutaneous T-cell lymphoma (CTCL) and have also demonstrated some therapeutic benefit in other malignancies. The approval of the HDAC inhibitor vorinostat (Zolinza™) was based on the inherent sensitivity of this type of lymphoma to alterations in acetylation patterns that resulted in the induction of repressed apoptotic pathways. However, the full potential of these inhibitors (epigenetic modulators) is still on the horizon, as the true breadth of their utility as anti-cancer agents will be determined by the careful analysis of gene expression changes generated by these inhibitors and then combined with conventional chemotherapy to synergistically improve response and toxicity for an overall enhanced therapeutic benefit to the patient. The question that must be considered is whether the current HDACIs are being utilized to their fullest potential in clinical trials based on their mechanism-based alterations in disease processes.

1. Histone deacetylase inhibitors

The role of gene regulation by physical alterations of either DNA or the structural components of chromatin has recently been highlighted as a major process in neoplastic transformation and maintenance of the malignant phenotype. The discovery that chromatin contains a dynamic group of nuclear proteins that regulate transcription of many genes and especially some tumor suppressor genes came about with the discovery that the histone deacetylases (HDACs) were the target for a potent natural product that induced differentiation of neoplastic cells [1]. Several other compounds were initially discovered as inducers of differentiation and as mimetics of growth factor pathways (TGFβ) that were subsequently shown to have a mechanism of action that involved inhibition of histone deacetylase enzymes [2–7]. Recently, there have been several excellent reviews of the HDAC field both preclinical characterization of histone deacetylase inhibitors (HDACIs) and clinical development of HDACIs [8–11]. A brief overview of the mechanism of HDACIs and their road to the clinic is warranted here.

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doi:10.1016/j.bcp.2007.04.007

Regulation of gene transcription occurs by various mechanisms including (1) DNA methylation, (2) post-translational histone modifications (primarily acetylation but also includes methylation, phosphorylation, poly-ADP-ribosylation, ubiquitinylation, sumoylation, carbonylation and glycosylation), and (3) RNA-associated silencing [8,10]. It has long been recognized that neoplastic cells exhibit aberrant gene expression; therefore, strategies have been investigated to correct these genetic perturbations through pharmacological manipulation of the epigenome, namely, modulation of DNA methylation and histone acetylation. As the most promising clinical data have emerged from modulation of histone acetylation the focus here will be on this unique balance that maintains nucleosomal DNA in either an active (open, acetylated) or inactive (closed, deacetylated) form. This balance is controlled by the reciprocal activities of the acetylating enzymes, histone acetyltransferases (HATs) and deacetylating enzymes, histone deacetylases (HDACs). Epigenetic alterations are crucial to the onset and progression of cancer, and HDACIs have been demonstrated to reverse some of the aberrant epigenetic states associated with cancer through induction of hyperacetylation of nucleosomal histones resulting in expression of repressed genes that produce growth arrest, terminal differentiation, and/or apoptosis in carcinoma cells.

HDACs were first identified as the target of a natural product, trapoxin, which caused differentiation of cancer cells and was used as an affinity ligand to pull out the target, HDAC1, from the cell lysate [1]. Other various differentiation assays or promoter/reporter construct assays for the TGFβ pathway identified other compounds whose mechanism of action was later elucidated to be inhibition of HDACs. The biological activity of compounds such as the spiruchostatins A and B [6], diheteropeptin [7,12], scriptaid, and A-161906 were all discovered using these assays and subsequently, the mechanism of action of all these compounds was determined to be inhibition of HDACs. These observations emphasize the prominent role of HDACs in the signaling pathways regulated by TGFβ and how modulation of chromatin structure can produce desired pharmacological effects.

1.1. *Deacetylase enzymes—the HDAC family*

The HDACs can be divided into two families, (1) the Zn^{+2}-dependent HDAC family composed of class I (HDACs 1, 2, 3 and 8), class II a/b (HDACs 4, 5, 6, 7, 9 and 10), and class IV (HDAC 11) and (2) Zn^{+2}-independent NAD-dependent class III SIRT enzymes (Table 1). The class I HDACs, apparently the "true" histone deacetylases, are localized to the nucleus of cells. The classes II a/b deacetylases have both histones and non-histone proteins as substrates and are primarily localized to the cytoplasm but are known to shuttle in and out of the nucleus through association with 14-3-3 proteins. The class II enzymes are characterized by either a large N-terminal domain or a second catalytic domain (e.g., HDAC 6 which contains both a histone and a tubulin deacetylase catalytic domain). The class III SIRTs are NAD^+-dependent deacetylases with non-histone proteins as substrates (in mammalian cells) and have been linked to regulation of caloric utilization of cells (only in yeast are the SIR proteins known to be histone deacetylases) [8]. HDACs do not function independently but rather in concert with multi-protein complexes (e.g., NCoR, SMRT, MEF, MeCP2, Sin3A, etc. [13]) that are recruited to specific regions of the genome that in turn generate the unique spectrum of expressed and silenced genes that are characteristic of the expression profile(s) responsible for the malignant phenotype of cancer cells.

Table 1 – HDACs

HDAC	~MW[a] human	~MW[a] murine	AAs[b] human	AAs[b] murine	%Similarity to human (nucleic acid/amino acid)
Zn^{+2}-dependent					
Class I					
1	55,103	55,076	482	482	90.8/99.4[c]
2	55,364	55,331	488	488	91.1/98.6[c]
3	48,848	48,821	428	428	92.5/99.6[c]
8	41,758	41,772	377	377	90.9/96.3[c]
Class IIA					
4	119,070	118,562	1084	1076	86.3/94.2
5	121,992	120,941	1122	1113	91.1/95.6
7	102,927	101,286	952	938	86.8/90.3
9	111,297	65,631	1011	588	90.394.8
Class IIb					
6	131,431	125,703	1215	1149	81.1/78.7[d]
10	71,445	72,111	669	666	78.1/76.4[d]
Class IV					
11	39,183	39,157	347	347	87.3/91.9

[a] Estimated molecular weight based on amino acid sequence, may be different than observed molecular weight on SDS-PAGE gels.
[b] AAs—number of amino acids in the open reading frame.
[c] Class of HDACs with the greatest similarity between murine and human species.
[d] Class of HDACs with the greatest difference between human and murine species.

1.2. *Which HDACs are responsible for aberrant transcription?*

One key question relates to which of the HDAC(s) are important to inhibit to obtain the desired pharmacological profile for the therapy of cancer. Genetic studies, knockout in yeast and siRNA in mammalian cells, have indicated that the class I HDACs are essential to cell proliferation and survival [14,15]. However, few studies have addressed the *in vitro* HDAC enzyme selectivity of low molecular weight HDAC inhibitors between classes I and II HDACs, most likely due to the difficulty in obtaining isolated isozymes free of other HDACs [16,17]. As described above, the HDACs function in complexes with other co-repressor proteins and in concert with DNA and histone methylation; therefore, one should exercise caution in the strict interpretation of results with siRNA knockdown or any other type of genetic knockdown/knockout that eliminates these proteins entirely from the complex and may therefore have effects other than those observed when using small molecule inhibitors. Minucci and Pelicci [11] state "... no conclusive experimental evidence that points to specific HDACs as being selectively involved in any form of disease, including cancer." However, experiments have demonstrated that HDAC1 knockout (recombinant) is embryonic lethal, HDAC2 knockdown by siRNA regulates cell survival [11], and that siRNA knockdown of HDACs 1 and 3 (class I) but not 4 and 7 (class II) results in an antiproliferative phenotype [18]. All these data suggest the role of class I HDACs in cancer cell proliferation and survival and that dysregulation of their normal function is potentially a driving force in neoplastic transformation and progression.

So exactly how do HDACIs cause cancer cell death? As with most pharmacological agents, the type of cell death induced by HDACIs can be cell type dependent and context dependent. Several recent reviews have addressed this issue and have detailed each possible mechanism [8,10]. One assumption is that cell death is caused by the re-expression of repressed genes upon HDACI treatment; however, HDACIs have the ability to induce cell death by other mechanisms independent of re-expression of genes. It has also been noted that combination of epigenetic inhibitors (DNA methylation inhibitors and HDACIs) results in synergistic cell death and it is yet unclear if this synergism reflects the re-expression of silenced genes or potentiation of cell death through acetylation of non-histone proteins [10]. HDACI mediated cell lethality can be generalized into several different mechanisms: (1) acetylation and disruption of the activity of client proteins for the heat shock proteins; (2) perturbation of the NFκB pathway; (3) up-regulation and activation of the extrinsic apoptotic pathway (death receptor pathways); (4) induction of oxidative injury (ROS); (5) generation of pro-apoptotic second messengers such as ceramide. Again the data clearly suggests that the mechanism actually causing cancer cell death is very cell type specific. Understanding which of these mechanisms is operative in a specific cancer type and which HDAC(s) are responsible may be critical to optimizing their rational incorporation into combination regimens [10].

2. HDAC inhibitors in clinical development

HDACIs currently in clinical development cover pan-HDACIs (vorinostat, belinostat, and LBH589) and somewhat isotype selective agents (romidepsin, MS-275 and MGCD0103) (Tables 2 and 3). With the approval of Zolinza (vorinostat, SAHA) by the FDA for the treatment of CTCL and with other histone deacetylase inhibitors awaiting approval for various cancers, this will hopefully prompt the investigation of histone deacetylase inhibitors into a broader range of disease states where altered chromatin function may play a role in their pathophysiology (see Section 3.7).

2.1. *Vorinostat (SAHA, Zolinza™): clinical update*

The sensitivity of CTCL cells to vorinostat was demonstrated in cell lines (Hut78, HH and MJ cells) and in primary peripheral blood lymphocytes from CTCL patients [19]. In normal PBLs vorinostat increased apoptosis from 6 to 13% whereas in CTCL patient PBLs vorinostat increased apoptosis from 15 to 32%, suggesting selectivity of vorinostat (HDACIs) for malignant versus normal cells. Vorinostat increased the acetylation of histones H2B, H3 and H4 and also increased the expression of p21 and Bax while decreasing the expression of STAT6 and decreasing levels of phospho-STAT6; all ultimately leading to the activation of caspase 3, cleavage of PARP and apoptosis [19]. Similar results have been seen for romidepsin (depsipeptide, FK228) in Hut78 cells [20].

Generally, vorinostat has been well tolerated in Phase I studies administered either i.v. or orally. The dose limiting toxicities (DLTs) observed in these studies included gastrointestinal (nausea, vomiting and/or diarrhea), anorexia, dehydration, fatigue and myelosuppression (thrombocytopenia,

Table 2 – HDAC inhibitors in clinical development

HDACI	Zn^{+2}-chelator[a]	Selectivity[b]	Clinical development
Zolinza™, vorinostat, SAHA	HA	Pan	Approved
LBH 589	HA	Pan	II/III
Belinostat, PXD101	HA	Pan	I/II
Romidepsin, depsipeptide, FK228	SH	Class I	I/II
MS-275	AN	Class I	I/II
MGCD0103	AN	Class I	I/II

[a] HA: hydroxamic acid; SH: sulfhydryl; AN: anilide.
[b] Pan: inhibitor of both classes I and II HDACs (enzyme and cellular); class I: inhibitor of primarily class I HDACs (enzyme) or compounds that lack inhibition of α-tubulin deacetylation in cells.

Table 3 – Ongoing clinical trials with HDACIs (www.ClinicalTrials.gov)

Clinical trial	Sponsor	Clinical phase	Description
MGCD0103			
NCT 00323934	Pharmion/MethylGene	I	3x/wk oral, advanced solid tumors or non-Hodgkins lymphoma
NCT 00324194		I	2x/wk oral, leukemia and MDS
NCT 00324129		I	3x/wk oral, leukemia and MDS
NCT 00359086		II	3x/wk oral, relapsed and refractory lymphoma
NCT 00358982		II	Relapsed and refractory Hodgkins lymphoma
NCT 00374296		II	AML/high risk MDS in the elderly and previously untreated or adult w/relapsed/refractory disease
NCT 00372437		I/II	Combination with gemcitabine (Gemzar) in refractory solid tumors/MG0103-Gemzar to Gemzar naïve patients w/locally advanced (non-resectable stage III) or metastatic (stage IV) pancreatic cancer
NCT 00324220		I/II	Combination with Vidaza (azacitidine) in high risk MDS or AML
Belinostat (PXD101)			
NCT 00413075	CuraGen Corp.	I	Oral PXD101 in advanced solid tumors
NCT 00336804	U. Chicago/NCI	I	Oral, combination w/azaditidine in AML, ALL, APL, CML and MDS
NCT 00274651	CuraGen Corp.	II	Oral, CTCL, PTCL, non-Hodgkins lymphoma
NCT 00411476	MD Anderson/CuraGen Corp.	I	Oral, QD or BID in advanced solid tumors
NCT 00413322	CuraGen/TopoTarget A/S	I	Combination w/5-FU in advanced solid tumors and advanced colorectal cancer
NCT 00348985	U. Colo./NCI	I	Combination w/bortezomib (PS-341) in advanced solid tumors and lymphoma, i.v. day 1–5 30 min infusion
NCT 00421889	CuraGen/TopoTarget A/S	I/II	Belinostat (PXD101) combination w/carboplatin or paclitaxel in advanced solid tumors—ovarian, epithelial ovarian and fallopian tube cancers
NCT 00351975	U. Chicago/NCI	I	Combination w/azacitidine (5-aza) in advanced hematological malignancies, CML, leukemia, MDS or MPD
NCT 00334789	CA Cancer Consortium/NCI	I	Combination w/isotretinoin in metastatic or unresectable solid tumors
NCT 00357162	Mayo Clinic/NCI	II	MDS (i.v. days 1–5 30 min infusion)
NCT 00357032	CA Cancer Consortium/NCI	II	Relapsed or refractory AML or older patients w/newly diagnosed AML (i.v. days 1–5 30 min infusion)
NCT 00321594	Cancer Therapeutics Research Group/NCI	I/II	Liver cancer, unresectable HCC
NCT 00303953	SWOG/NCI	II	Relapsed or refractory aggressive B-cell non-Hodgkins lymphoma
NCT 00965053	CA Cancer Consortium/NCI	II	Malignant mesothelioma
NCT 00301756		II	Platinum-resistant epithelial ovarian cancer and micropapillary/borderline (LMP) ovarian tumors
NCT 00354185	U. Wisconso/NCI	I	Combination w/17-AAG in metastatic or unresectable tumors or lymphoma
LBH 589			
NCT 00419536	Novartis	I	Single agent and combination w/docetaxel and prednisone (oral)
NCT 00412997	Novartis	I	Advanced solid tumors or CTCL (oral)
NCT 00425555	Novartis	II/III	CTCL (refractory)
Romidepsin (depsipeptide)			
NCT 00106431	Gloucester Pharm.	II	CTCL
NCT 00379639	Gloucester Pharm.	I/II	Combination w/gemcitabine in pancreatic cancer
NCT 00299351	Royal Marsden NHS Foundation Trust	II	PTCL patients who have completed a prior clinical study w/FK-228
NCT 00112463	Wake Forest Univ./NCI	II	Metastatic or unresectable soft tissue sarcoma

NCT 00084682	Albert Einstein Coll. Of Med./NCI	II	Unresectable, recurrent or metastatic squamous cell carcinoma of the head and neck
NCT 00079443	U. Maryland Greenebaum Cancer Center/NCI	I/II	Combination w/rituximab and fludarabine in relapsed or refractory low grade B-cell non-Hodgkins lymphoma
NCT 00098644	NCI	I	Combination w/flavopiridol in advanced lung, esophageal or pleural caner
NCT 00085540	North American Brain Tumor Consortium/NCI	I/II	Recurrent high-grade glioma
NCT 00052767	NCI	I	Refractory thyroid or other advanced solid tumor
NCI 00383565	MD Anderson/NCI	II	Relapsed or refractory non-Hodgkins lymphoma
NCT 00020436	NCI	II	CTCL and relapsed PTCL
NCT 00041158	NCI	I	Combination w/decitabine in unresectable advanced lung, esophageal, pleural mesothelioma, or lung metastases
MS-27-275			
NCT 00387465	Sidney Kimmel Comp. Cancer Ctr./NCI	I/II	Combination w/5-azacitidine in recurrent advanced non-small cell lung cancer
NCT 00101179	Sidney Kimmel Comp. Cancer Ctr./NCI	I	Combination w/5-azacitidine in MDS, CMMoL and AML
NCT 00313586	ECOG/NCI	II	Combination w/5-azacitidine in MDS, CMMoL and AML
Vorinostat (SAHA)			
Ongoing studies—41		I–III	Combinations w/tamoxifen, bortezomib, temozolomide, doxorubicin, idarubicin, azacitidine, isotretinoin, cytarabine/etoposide, decitabine, flavopiridol, trastuzumab, capecitibine, bevacizumab, 5-FU, leucovorin, oxaliplatin, gemzar, paclitaxel/bevacizumab, tagretin, carboplatin/paclitaxel

neutropenia and/or leukopenia) [21]. HDAC inhibition was demonstrated in patients as increased accumulation of acetylated histones in tumors, bone marrow and peripheral blood cells. Phase I studies with vorinostat have resulted in complete and partial responses (CRs and PRs, respectively) in both refractory solid and hematological malignancies. The major adverse events (AEs) observed with vorinostat differ by route of administration, i.v. or oral, possibly due to differences in pharmacokinetics; oral vorinostat produced fatigue, diarrhea, anorexia and dehydration as major AEs, whereas i.v. vorinostat produced myelosuppression and thrombocytopenia as major AEs [22]. Clinical improvement was observed in Phase I studies with vorinostat in renal cell carcinoma, head and neck squamous carcinoma, mesothelioma, B- and T-cell lymphomas and Hodgkins disease [23].

In a Phase II study of vorinostat in CTCL no CRs were observed and 8 out of 33 patients achieved PRs (1 early stage disease and 7 advanced stage disease). Pruritis is a major clinical symptom of CTCL and 14 out of 31 patients (45%) with baseline pruritis had symptomatic relief. Histologically in these CTCL patients a significant decrease in dermal vessel density occurred after 4 weeks of therapy (11 out of 18 or 61%) and correlated with an increase in thrombospondin-1 (TSP-1, a known antiangiogenic protein) expression in the dermis. Overall in this Phase II study there was a 24% response rate in a heavily pretreated and refractory patient population where 8 out of 33 patients achieved PRs and 11 out of 33 patients had significant relief of pruritis and/or SD, providing for an overall clinical benefit in 58% of these patients [21].

The Phase IIb of vorinostat in CTCL was an open label 400 mg once daily dose with an overall response rate of 27%. There was an 81% reduction in SWAT scores and a 32% decrease in overall pruritis with a 43% improvement in severe pruritis. Fifteen out of 74 patients have been treated for >1–2 years. Vorinostat has a medium time to response of 55 days and maximum duration of response has not been reached with patients now beyond 448 days of treatment. Fatigue and GI were the most common AEs and Grades 3–4 thrombotic events occurred in 5% of the patients (4 out of 74). Patient PBMCs were analyzed by gene array and 2 h after a dose there were decreases in genes associated with proliferation and an increase in genes associated with apoptosis. Proteinuria was only seen as a Grades 1–2 toxicity [24]. Zolinza was approved in 2006 for treatment of refractory CTCL and is currently in multiple clinical trials in combination with other chemotherapeutic agents (Table 3).

2.2. *Romidepsin (depsipeptide, FK228, FR901228): clinical update*

Romidepsin is a novel natural product bicyclic tetrapeptide HDACI [5]. Romidepsin was isolated from *Chromobacterium violaceum* and was found to reverse the transformed phenotype of Ha-Ras transformed cells and was antiproliferative in a wide variety of murine and human tumor cell lines both *in vitro* and *in vivo* [25]. Romidepsin is a pro-drug, the active moiety being a sulfhydryl group acting as the Zn^{+2}-chelator [26]. This pro-drug structure provides stability allowing both *in vivo* dosing and its use in humans [26]. As romidepsin is not a hydroxamic acid, and similar to other non-hydroxamates [27],

it is a more selective inhibitor of the class I HDACs (HDACs 1 and 2 versus HDACs 4 and 6) [26]. Romidepsin, possibly due to being a natural product tetrapeptide, is a substrate of MDR-1; however, cross-resistance has not been observed with other cytotoxic agents [28].

There have been multiple Phases I and II trials with romidepsin. Generally, romidepsin is well tolerated and has a similar toxicity profile as vorinostat. Two of the early Phase I trials demonstrated changes in the ECG of patients. DLTs observed with i.v. infusion on a 3- out of 4-week schedule consisted of fatigue, nausea, vomiting, and transient thrombocytopenia and neutropenia. The MTD was determined to be 17 mg/m^2 on days 1 and 5 every 21 days. In the Phase I studies cardiac arrythmias manifested by changes in the ECG were observed with a case of atrial fibrillation [29]. Acetylation of histones in patient's PBMCs was observed confirming inhibition of HDACs by romidepsin and there was one response out of 37 patients indicating limited activity in patients with solid tumors [29]. Another Phase I trial by the National Cancer Institute (NCI) demonstrated significant activity in CTCL patients with three PRs and one CR in a peripheral T-cell lymphoma (PTCL) [30]. The activity observed in this Phase I trial prompted the Phase II trial in CTCL.

Phase II clinical trials in T-cell lymphoma continued to demonstrate the efficacy of romidepsin in this disease. In patients with PTCL, 10 out of 27 patients demonstrated activity with a weekly infusion of romidepsin for 3 out of 4 weeks. In a recent Phase II study in CTCL and PTCL the overall response rate for CTCL was 31% with 3 CRs, 10 PRs and 9 SD, and for PTCL was 30% with 3 CRs and 8 PRs. An increase in histone acetylation was observed in normal and malignant blood cells and there was also an up-regulation of MDR-1 in these cells, consistent with *in vitro* findings [31]. In Phase II studies in solid tumors, romidepsin continued to demonstrate only marginal activity. These studies demonstrated the same DLTs as observed in the Phase I studies; fatigue, nausea and vomiting as well as myelosuppression and abnormalities in ECGs. A study of the cardiac risks in these patients determined that the ECG abnormalities were not indicative of myocardial dysfunction or myocardial damage. The clinical effect of the observed prolongation in the QTc interval on the safety profile of romidepsin is still under investigation [32]. Romidepsin is still undergoing multiple Phase II investigations to determine efficacy and the effect of QTc prolongation on the utility of this novel HDACI (Table 3).

2.3. MS-275: clinical update

MS-275 is a novel benzamide-based HDACI which like other non-hydroxamic acid inhibitors is somewhat selective for the class I HDACs [8,27,33]. MS-275 inhibits the proliferation of multiple carcinoma cell lines in the micromolar range and its mechanism of inducing cell death appears to involve generation of ROS [9,34]. MS-275 inhibits the growth of tumors implanted into mice in a dose-dependent manner and recently, through the use of a fluorescence-based gene expression reporter system based on the p21 promoter, it was demonstrated that a single dose of MS-275 can induce fluorescence in tumors in a time- and dose-dependent manner [35]. This reporter system demonstrated direct *in vivo* epigenetic regulation by this HDACI and can potentially be used to predict long-term anti-tumoral efficacy for MS-275 in animal models [35].

Phase I studies in advanced solid tumor and lymphoma patients demonstrated that the half-life of MS-275 was much longer than predicted based on preclinical models, 39–80 h in humans. Therefore, it is not surprising that a daily dose for 28 days on a 6-week schedule was not tolerated even at the initial dose of 2 mg/m^2 [36]. The MTD on a q14 day schedule was 10 mg/m^2 with DLTs of nausea, vomiting, anorexia and fatigue after oral dosing. In all these studies and all dose levels of MS-275, an increased acetylation of histone H3 in PBMCs was observed indicating that MS-275 was biologically active at the doses being evaluated [36]. In another Phase I study in adult refractory and relapsed acute leukemias, MS-275 had an MTD of 8 mg/m^2 on a weekly × 4 every 6-week schedule. In this study, DLTs were primarily infections and neurologic toxicity that included unsteady gait and somnolence, but also included fatigue, anorexia, nausea, vomiting, hypoalbuminemia and hypocalcemia [37]. In all dose groups as before, increased accumulation of acetylated histones H3 and H4, increased p21 expression and activation of caspases was observed in bone marrow mononuclear cells. However, in this study no responses based on classical criteria were observed [37]. MS-275 is currently undergoing Phase II studies in combination with 5-azacitidine in non-small cell lung cancer, MDS, CMMoL and AML (Table 3).

2.4. LAQ824, LBH589, belinostat, MGCD0103: clinical update

LAQ824 [38] and the more potent analog LBH589 are pan-HDAC inhibitors developed by Novartis. LBH589 and LAQ824 are unique in that they have been extensively studied for their role in the regulation of Hsp90 and degradation of Hsp90 client proteins [39]. Enhanced efficacy or synergy *in vitro* can clearly be demonstrated when LBH589 is used in combination with direct inhibitors of the client proteins of Hsp90, e.g., bcr-abl in CML [40,41] and FLT3 in AML [42]. HDAC inhibition results in the accumulation of acetylated Hsp90 and inhibition of its chaperone function with its client protein(s) results in ubiquitination and degradation of the client protein(s); therefore, the combination of a direct inhibitor of the enzyme (client protein) and decreasing client protein levels results in synergistic inhibition of the pathway and enhanced killing of those cells. Recently, LBH589 has also been demonstrated to deplete members of the polycomb repressive complex 2 (EZH2, SUZ12 and EED proteins) and DNMT1 in CML cells [43]. Currently, LBH589 is in Phase II/III clinical development in patients with CTCL (Table 3). At an MTD dose of 20 mg M, W, F, two patients achieved CRs, four attained PRs and one achieved SD for an overall response rate of 60% in patients with advanced-stage CTCL [44]. Multiple Phase I trials are ongoing with LBH589 and it is entering Phase II/III clinical studies (Table 3).

Belinostat (PXD101) is a novel hydroxamic acid HDACI (Table 2) and is cytotoxic to numerous cancer cell lines with IC_{50} values in the range of 0.2–3.4 μM [45,46], consistent with the activity of most other hydroxamate-based HDACIs. Belinostat has demonstrated *in vivo* activity against ovarian

and colon cancer xenograft models without significant toxicity in these murine tumor models. In ovarian cancer models, Belinostat demonstrated additive to synergistic activity when combined with standard cytotoxic agents such as carboplatin and paclitaxel [47]. Gene expression studies with belinostat and have identified regulation of target genes that should guide the selection of therapeutically effective combinations (e.g., 5-FU and thymidylate synthase as discussed below) [48]. Down-regulation of Aurora A and B kinases at the mRNA and protein levels was also observed which may contribute to the G2/M delay observed with belinostat [49]. The initial Phase I trial of belinostat was conducted in patients with advanced solid tumors. The most common adverse events were fatigue, nausea, vomiting and phlebitis (no grade 4 toxicities were observed in this initial Phase I trial). An MTD of 1000 mg/m^2/day was determined for progression into Phase II trials [50]. Interestingly, similar to vorinostat, belinostat did have 33% bioavailability when administered orally in these clinical trials. A Phase Ib study in colorectal carcinoma in combination with 5-FU is ongoing, no grade 3 or 4 toxicities have been observed at 1000 mg/m^2/day and the common adverse events include fatigue, nausea, vomiting, dysgeusia, dehydration and anorexia [51]. Currently multiple Phase I trials are ongoing in combination with agents such as Velcade and Vidaza (5-azacytidine) in multiple myeloma and hematological malignancies, respectively (Table 3). A Phase II study was recently reported in patients with advanced multiple myeloma who received monotherapy belinostat for >2 cycles (12 pts), there were six SD and six PD demonstrating that belinostat treatment resulted in stabilization of advanced and progressive disease. The combination of belinostat with dexamethasone (standard of care for MM patients) led to one MR as well as long duration of stable disease even in patients who have received multiple dexamethasone regimens [52]. Belinostat is currently in multiple Phase I/II clinical trials (Table 3).

MGCD0103 is a novel, orally bioavailable anilide-based HDACI developed by MethylGene, Inc. (http://www.MethylGene.com). This molecule is selective for the class I HDACs. This profile of HDAC inhibition has been determined by siRNA and antisense oligonucleotides to be optimal for inhibition of cell proliferation and survival. MGCD0103 is antiproliferative in a wide variety of liquid and solid tumor cell lines, causes the accumulation of acetylated histones (not α-tubulin), induces gene changes characteristic of other HDACIs and has been shown to enhance the activity of several different chemotherapeutics. MGCD0103 is in multiple Phases I and II clinical trials as a single agent or in combination with various chemotherapeutics including gemzar and Vidaza (azacitidine). Cancers being targeted by MGCD0103 include pancreatic (combination with gemzar), MDS and AML in combination with Vidaza, diffuse large B-cell lymphoma (DLBCL), follicular lymphoma, and relapsed or refractory Hodgkin's lymphoma. In a Phase I study in AML and MDS, as monotherapy, MGCD0103 was dosed twice weekly in a 3-week cycle, no MTD has been reached up to 66 mg/m^2/day. Non-dose-limiting toxicities included fatigue, nausea and vomiting. Inhibition of HDAC activity in PBMCs from the majority of treated patients was observed in this Phase I trial [53]. MGCD0103 is currently in multiple Phase I/II clinical trials (Table 3).

3. Mechanism-based potential of HDACIs: are HDACIs being utilized in combinations that make mechanistic sense to achieve optimal therapeutic potential?

Several factors enter into paradigms of therapeutic combinations with epigenetic modulators: first, and the first demonstrated utility of HDACIs [54], the presence of oncogenic fusion proteins that incorporate HDACs or make high affinity complexes with HDACs; second, what are the genes regulated by these agents and how are they regulated; third, are these direct effects on proteins involved in apoptosis or client protein stability; fourth, are these effects due to direct induction of oxidative injury in cells (Fig. 1). Several of these aspects are discussed below.

3.1. *Oncogenic fusion proteins that incorporate HDACs*

Hematological malignancies containing an oncogenic fusion protein were the first demonstration of the mechanism-based utility of HDACIs. Acute promyelocytic leukemia normally responds to retinoic acid (RA), inducing differentiation of the neoplastic cells and growth arrest. However, when the retinoic acid receptor (RARα) is expressed as a fusion protein with promyelocytic leukemia (PML) or promyelocytic leukemia zinc finger (PLZF), these cells are resistant to physiological levels of RA. In fact the PML fusion protein requires pharmacological doses of RA and the PLZF fusion protein with RARα does not respond at all to RA as a result of physical association of HDACs with these fusion proteins and repression of genes induced by RA that normally result in differentiation. In APL patients, treatment with HDACIs and RA is extremely effective in de-repressing RA target genes and inducing cellular responses to RA both *in vitro* and *in vivo* [11]. In at least one patient with the PLZF-RAR fusion protein, combination therapy of RA with the HDACI sodium phenylbutyrate generated disease remission [54]. This patient achieved a fourth CR that was sustained for several months [55]. When these types of fusion proteins are present, combinations with HDACIs has clear therapeutic potential for those patients.

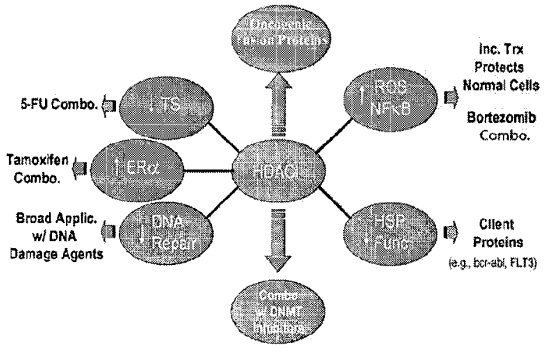

Fig. 1 – Avenues of epigenetic modulation with HDACIs based on known modulation of genes and pathways in cancer cells.

Several mutations (G289R and P407S) in the DNA binding domain of the PML-RARα also confers resistance to RA, which can be overcome by addition of a known HDACI [55]. These types of mutations occur frequently after multiple RA treatment relapses. The responses seen with RA plus sodium phenylbutyrate in these patients may be related to the potential of sodium phenylbutyrate to circumvent the blocked RA-regulated gene response pathway [55]. Therefore, alleviation of HDAC-induced repression restores normal function and differentiation capabilities ultimately leading to growth arrest and apoptosis in malignant cells.

So why with so much evidence of HDAC association with these oncogenic fusion proteins, is it that HDAC inhibitors have not performed as well as expected? Tabe et al. [28] demonstrated the critical importance of mechanism-based sequential therapy in future clinical trials that combine HDACI, ATRA and the anthracyclines; this apparently needs to be exploited clinically now that HDACIs are approved. Understanding the correct combination from a mechanistic viewpoint can lead to optimal tumor response, something not observed in the clinic in some forms of APL. Combining HDACIs with other types of agents that target co-repressor complexes therefore warrants further investigation to ultimately provide an optimal therapeutic response in these APL patients with RA non-responsive disease [10].

3.2. *Re-expression of the estrogen receptor-α (ERα)*

Yang et al. [56] described re-expression of the silenced ERα induced by TSA in MDA-231 ER-negative cells and implicated histone deacetylation as a critical component of ER gene silencing in human breast cancer cells. This observation suggested that activation of the silenced ER by various HDACIs could open a new avenue for therapy of a subset of advanced breast cancer with hormonal resistance [56]. Subsequently, the use of HDACIs to re-activate the silenced *ERα* gene demonstrated increased sensitivity of ER-negative cells to tamoxifen. It was also recognized that the *ERα* gene has a methylated promoter region in hormone resistant disease and the re-expression of the ER gene is enhanced by treatment with a HDACI and a methyltransferase inhibitor, 5-aza-2′-deoxycytidine [57]. Studies with LBH589 in MDA-231 and MDA-435, ER-negative breast cancer lines, demonstrated that ERα could be reactivated without demethylation of the ER promoter [58]. It was also demonstrated that LBH589 released DNMT1, HDAC1 and SUV39H1 (a histone demethylase) from the ER promoter; this was associated with an increased acetylation of histones H3 and H4, a decrease in methylation of histone H3-K9, and impaired binding of heterochromatin protein 1 (HP1 alpha). The re-activation of ERα by LBH589 was sustained for 96 h after withdraw of the HDACI. LBH589, as with other HDACIs, enhanced 4-hydroxy-tamoxifen sensitivity in MDA-231 cells [58]. Re-expression of ERα in MDA-231 cells with the combination of TSA and 5-aza-2′-deoxycytidine (5-aza-dC) restored tamoxifen sensitivity and also recruited distinct co-repressor complexes to the tamoxifen bound ERα to form a repressive ER complex at ER target genes, turning off these ER responsive genes. The complex was characterized by the presence of recruited HDAC3/NCoR and TBL1 [59]. Interestingly, in ER-positive breast cancer cells (MCF-7), ERα is actually repressed by HDACIs and results in an antiestrogenic effect. This effect on ERα, which also includes pS2 and cyclin D1, is mediated by the recruitment of the methylated DNA binding protein, MeCP2, and the exclusion of DNMT1 from the promoter regions by agents such as valproic acid [60].

Here is an example where use of an HDACI as monotherapy would probably not produce a clinical benefit to the patient; however, when combined appropriately based on the response to HDAC inhibition (with or without DNMT inhibition), re-expression of ERα may lead to significant clinical responses in a subset of refractory advanced cancer patients. Clinical trials combining vorinostat with tamoxifen are on going to investigate the clinical potential of this combination (Table 3).

3.3. *Regulation of DNA repair by HDACIs*

It has been demonstrated that all HDACIs to date synergize with ionizing radiation (γ-irradiation) to kill tumor cells *in vitro* and several have shown this synergy *in vivo* [8]. In the absence of double strand DNA breaks caused by radiation, the HDACIs can independently induce or mimic the DNA damage response by activation of ATM which phosphorylates and activates downstream effectors such as BRCA1, 53BP1, CHK2 and γ-H2AX, ultimately leading to apoptosis. One mechanism where HDACIs can synergize with radiation is through down-regulation of the genes/proteins involved in the DNA damage response such as Ku70, Ku80, Rad50 and DNA ligase IV [8,61]. By reducing the proteins responsible for repairing the DNA damage due to ionizing radiation, the greater the overall DNA damage and the greater the apoptotic response. It has also been demonstrated that HDAC4 is recruited to DNA-damage-induced foci and co-localizes with 53BP1 in irradiated cells. Knockdown of HDAC4 by siRNA decreased levels of 53BP1, abrogated the DNA damage-induced G2 delay, and sensitized HeLa cells to radiation-induced apoptosis [62]. In addition to reducing the expression of some of the DNA damage-induced response genes, HDACIs (e.g., vorinostat) in combination with radiation increase the relative number of γ-H2AX foci in cells, which can potentially be used as a predictive marker of radiotherapy response to vorinostat [61].

This is a common mechanism exploited by combinations with HDACIs, reduction of repair or survival proteins, resulting in synergistic killing of tumor cells in combination with many of the classical chemotherapeutic compounds now used to treat cancer. Methylation and repression of the DNA mismatch repair gene hMLH1 results in resistance to cisplatin treatment. In this case, HDACIs in combination with DAC (2-deoxy-5-azacytidine) synergize to re-express hMLH1 and sensitize cells to cisplatin treatment. These results suggest that the combination of DNMT inhibitors and HDACIs could act synergistically to increase the efficacy of chemotherapy in patients that lack MLH1 expression due to MLH1 promoter hypermethylation [46]. Depending on the tumor type and the combination therapy being employed, HDACIs can either be used to induce pro-apoptotic proteins or reduce the enzymes that commonly repair damage induced by chemotherapeutic agents, again resulting in a more pronounced apoptotic response. Therefore, with the exception of CTCL where HDACIs have a profound direct effect on pro-apoptotic genes,

why would investigators be considering monotherapy for HDACIs when their greater promise lies in combinations based on the mechanism of HDAC inhibition in those particular tumor cells?

3.4. *Reduction of chemotherapy target proteins: thymidylate synthase*

Thymidylate synthase (TS) is the target of the chemotherapeutic agent 5-fluorouracil (5-FU). Resistance to 5-FU is a common reason for treatment failure, especially in the treatment of colorectal cancers [63]. Resistance to a chemotherapeutic can occur by several mechanisms in cancer cells: up-regulation of metabolizing enzymes or drug pumps such as MDR-1, up-regulation of the downstream effectors of the target protein requiring less of a initial signal to evoke a response and/or the up-regulation of the target protein itself therefore require increasing concentrations of the chemotherapeutic to obtain the desired pharmacological effect. It has been recognized that gene amplification of TS with consequent increases in TS mRNA and protein results in an acquired resistance to 5-FU and fluorodeoxyuridine (FUDR) [63]. In patients treated with 5-FU an improved response was observed in those patients with low tumoral TS expression, and high TS expression predicted a poor response to 5-FU-based chemotherapy.

Therefore, one potential to sensitize cells to 5-FU would be to lower the concentration of its target protein, TS, both in non-resistant cancer cells and those with acquired resistance to 5-FU treatment. Initial gene expression studies with HDACIs recognized that TS was one of the HDACI gene targets [64]. Addition of an HDACI to 5-FU treatment of cells results in the synergistic killing of those cancer cells [48,65,66]. It has also been demonstrated that knockdown of TS by antisense oligonucleotides decreases tumor cell growth and reduces drug resistance [67], and this has been demonstrated *in vivo* using cells containing a full-length antisense construct for TS [68]. Connection of the two observations, knockdown of TS by antisense [67] or by HDACI [64], could be used to enhance the chemosensitivity of resistant and non-resistant cancer cells to 5-FU and was only recently investigated with HDACIs [69]. The enhanced sensitivity of cells to 5-FU after TSA treatment was related to the repression of TS mRNA and protein synthesis [49,69]. However, these investigators demonstrated that like several other targets of HDACI, the decrease in protein could also be accomplished by regulation of Hsp90 function, and TS is a client protein of Hsp90. Therefore, there are multiple mechanisms by which HDACIs can regulate the expression of a protein, at the level of mRNA production and stability of the protein by altering chaperone protein function. The conclusion of these studies like the others done with HDACIs and 5-FU is that this combinatorial approach may be useful to overcome 5-FU resistance [69]. Vorinostat and belinostat are currently in clinical trials in combination with 5-FU recognizing that HDAC inhibition results in repression of TS in solid tumors [51].

3.5. *Acetylation of non-histone proteins: regulation of Hsp90 function*

It was demonstrated with romidepsin that treatment of cells results in the reduction of p53, Raf-1 and ErbB, all client proteins of Hsp90; therefore, it was investigated whether romidepsin would affect Hsp90 function similar to 17-AAG which also causes a reduction of these proteins [39]. It was also demonstrated that the binding of mutant p53 and Raf-1 to Hsp90 was inhibited by treatment with romidepsin and that this inhibition was associated with acetylation of Hsp90. Similarly, the hydroxamic acid HDACI LAQ824 reduced the levels of bcr-abl (also a client protein of Hsp90) in CML cells. This reduction in bcr-abl protein sensitized these cells to imatinib (Gleevec). The repression of bcr-abl was protein synthesis inhibitor sensitive suggesting that LAQ824 augments the level and activity of a transcriptional repressor for bcr-abl. Interestingly, the promoter region for bcr-abl was not acetylated on histones H3 or H4 after treatment with LAQ824, this is quite unlike the promoter region for p21 that is hyperacetylated upon HDACI treatment corresponding to its up-regulation. Treatment of CML cells with the HDACI LAQ824 was associated with the induced acetylation of Hsp90, suggesting that LAQ824 through Hsp90 acetylation disrupts the stable chaperone association of bcr-abl with Hsp90 therefore promoting proteosomal degradation of bcr-abl [40]. This phenomenon was also observed in AML cells with the FLT3 kinase inhibitor PKC412 where LAQ824 induced Hsp90 acetylation, resulted in proteosomal degradation of FLT3 and decreased P-FLT3, P-STAT5, P-AKT and P-ERK1/2 levels, and synergistic apoptosis in MV4-11 cells (AML cell line containing the FLT3-ITD mutant) and primary AML cells expressing mutant FLT3 [42]. Co-treatment of CML cells with either LBH589 or vorinostat and either AMN 107 [41] or dasatinib [70], respectively, led to decreased levels of bcr-abl and the gleevec-resistant mutants (bcr-abl T315I and E255K) with concomitant decreases in P-STAT5, P-ERK 1/2, Bcl-xL, P-CrkL and increases in p27 and Bim, a pro-apoptotic protein [41,70]. HDACIs regulate many of the client proteins of Hsp90 by inducing the acetylation of Hsp90; other epigenetic regulators such as DMNT1 can be regulated both at this post-translational step or at the transcriptional step as stated above [43].

The ability of HDACIs to modulate Hsp90 function should be investigated fully to determine the breadth of client proteins affected by this mechanism and used in combination with inhibitors of these target proteins to obtain optimal synergistic killing of cancer cells. It is also of interest that in many of these cell types, the HDACIs also have a direct effect on the transcription of the client protein itself, thus adding a potential benefit to the use of HDACIs in cancers expressing these targeted client proteins. Another question posed by this data is which mechanism is dominant or responsible for the observed pharmacological effects of these HDACIs? Further investigations are clearly necessary to understand this phenomenon and determine the most beneficial utility of these HDACIs in relation to the client proteins being targeted.

3.6. *Methylation: DNA, histones, and other proteins*

Many of the strategies targeting epigenetic changes that drive malignant progression or tumorgenesis are combinations of an HDACI with a DNA methyltransferase (DNMT) inhibitor. Hypermethylation of specific promoter regions is known to silence genes and some cells use both hypermethylation and

repression by the incorporation of HDACs into co-repressor complexes to re-enforce gene silencing. Therefore, either an HDACI or DNMT inhibitor alone does not produce profound efficacy, but the combination of these two mechanisms sometimes generates a synergistic reactivation of specific genes and the desired pharmacological effect. It has become clearly evident that not only DNA methylation controls gene expression but also methylation of specific histone residues and other proteins/transcription factors. The histone code was described as a signaling mechanism through post-translation modification of histones (primarily acetylation, methylation and phosphorylation) required for the recruitment of the necessary components to repress or activate gene transcription [71–74]. DNA methylation is carried out by the DNA methyltransferases DNMT 1, 2 and 3a and 3b; histone methylation is carried out by the histone methyltransferases (HMTs) such as Suv4-20 and SET proteins [75]; methylation of transcription factors such as p53 is carried out by the Smyd family of proteins [76]. Many of the epigenetic genes regulating methylation or responses to methylated proteins are either over-expressed or severely dysregulated in many types of cancer [75].

The original dogma that implied histones were only static, structural proteins is now clearly not the case, this may now apply to methylation pathways. That is, methylation of DNA, histones and non-histone proteins has turned out to be a very dynamic process that modulates how cells respond to different stimuli (positive or negative). Methylation, like histone acetylation, involves the balance of methyltransferases and demethylation enzymes (of which only a few have been described to date and include the LSD-1 and JmjC protein families [77]). The approval of Vidaza (5-azacytidine) has validated the use of DNMT inhibitors for the treatment of myelodysplastic syndromes including chronic myelomonocytic leukemia and it is now in clinical trials combined with various HDACIs such as sodium phenylbutyrate, valproic acid and Zolinza (vorinostat). Again, once thought a rather static post-translational modification, DNA methylation is a dynamic process that not only controls such cellular functions as imprinting but also regulates active gene transcription and is a major contributor to tumorgenesis and malignant progression.

3.7. *Other diseases that may be treated with HDACIs*

Beyond cancer there may be several novel therapeutic arenas where epigenetic modulators may provide therapeutic benefit. Early in the investigations on HDAC inhibitors it was recognized that one of the effects was the activation of latent viruses (e.g., HIV). As this may seem problematic in the development of HDACIs, this mechanism can be utilized to reactivate a latent virus thus making it susceptible to subsequent treatment with targeted anti-viral therapy. This concept has been validated in humans with valproic acid, the HDACI, and highly active antiretroviral therapy (HAART) where resting cell infection declined significantly in three out of four patients treated with this combination [78,79]. HDACIs have effects on the acetylation of key factors that regulate immune cell function, such as STAT1, STAT3 and NFκB, and acetylation is known to regulate the function of these inflammatory transcription factors. HDACIs can reduce graft-versus-host disease following bone marrow transplantation by suppressing pro-inflammatory cytokines such as TNFα and IL-1 [8]. Similarly, with concentrations of HDACIs lower than those expected to have an antiproliferative effect, one can demonstrate alteration of genes during the inflammatory response. These gene changes effectively block the Th1 type but not the Th2 type inflammatory response. The genes modulated in monocytic and dendritic cells were fairly specific, not global, and in doing so provide abundant new targets for pharmacological intervention in inflammatory diseases [80]. HDACIs can also suppress the expression of key adhesion molecules such as VCAM-1, thus leading to a reduction in the number of activated monocytes binding to inflamed endothelium. This is yet another mechanism, selective inhibition of adhesion molecules, whereby HDACIs can have an anti-inflammatory effect [81]. In neurodegenerative states, HDACIs increase the expression of neuroprotective proteins such as Hsp70 and Bcl-2 in the ischemic brain and point to the therapeutic potential of HDACIs in stroke [82]. In other neurodegenerative diseases, dysregulation of transcription occurs through the inappropriate utilization of HATs and HDACs. HDACIs have shown activity in these diseases *in vitro* and in animal models of disease such as Huntington's disease, where expression of the mutant huntington protein displaces co-activators or directly binds and inhibits HATs resulting in the transcriptional repression observed early in the Huntington's disease process in animal models [83,84]. HDACIs therefore have the potential to reverse this disease process by re-expression of these silenced genes.

4. Summary

With the approval of vorinostat for the treatment of CTCL and PTCL, the application of epigenetic regulation as an avenue in treatment has expanded, not only for hematological malignancies, but also to a much broader range of cancers. The response rates in CTCL are impressive and the side effects are manageable. The greatest utility of these epigenetic modulators will be in combination with other therapeutics that synergize with the regulation being controlled by the epigenetic modulator. Only in this manner of combination will there be a sufficient response rate in solid tumors. These experiments are now ongoing in clinical trials of vorinostat, romidepsin, belinostat and LBH589.

As discussed herein, these are a few of the different disease that may benefit therapeutically with the use of HDACIs. One of the major issues still remaining is which of the HDACs are primarily responsible for the manifestation of the disease and can selective inhibitors be developed to address these specific diseases. It is clear that somewhat selective agents can be developed that distinguish at least the histone and non-histone protein deacetylation process [27,85]. As these more selective agents advance into clinical trials their utility will become apparent, but as discussed, their approval (efficacy in a disease state) may depend on effective combinations with other therapeutics to maximize the desired pharmacological benefit.

REFERENCES

[1] Taunton J, Hassig CA, Schreiber SL. A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p. Science 1996;272(5260):408–11.
[2] Glaser K, Li J, Aakre M, Morgan D, Sheppard G, Stewart K, et al. Transforming growth factor β mimetics: discovery of 7-[4-(4-cyanophenyl)phenoxy]-heptanohydroxamic acid, a biaryl hydroxamate inhibitor of histone deacetylase. Mol Cancer Ther 2002;1:759–68.
[3] Su G, Sohn T, Rhyu B, Kern S. A novel histone deacetylase inhibitor identified by high-throughput transcriptional screening of a compound library. Cancer Res 2000;60: 3137–42.
[4] Sohn T, Su G, Ryu B, Yeo C, Kern S. High-throughput drug screening of the DPC4 tumor-suppressor pathway in human pancreatic cancer cells. Ann Surg 2001;233(5): 696–703.
[5] Nakajima H, Kim Y, Terano H, Yoshida M, Horinouchi S. FR901228, a potent antitumor antibiotic, is a novel histone deacetylase inhibitor. Exp Cell Res 1998;241:126–33.
[6] Masuoka Y, Nagai A, Shin-ya K, Furihata K, Nagai K, Suzuki K-i, et al. Spiruchostatin A and B, novel gene expression-enhancing substances produced by *Pseudomonas* sp. Tet Lett 2001;42:41–4.
[7] Masuoka Y, Shin-Ya K, Furihata K, Hayakawa Y, Seto H. A novel substance with TGF-β like activity, diheteropeptin, produced by a fungus, *Diheterospora* sp. J Antibiot 1997;50(12):1058–60.
[8] Bolden J, Peart M, Johnstone R. Anticancer activities of histone deacetylase inhibitors. Nat Rev Drug Discov 2006;5:769–84.
[9] Rosato R, Grant S. Histone deacetylase inhibitors in clinical development. Exp Opin Investig Drugs 2004;13(1):21–38.
[10] Rosato R, Grant S. Histone deacetylase inhibitors: insights into mechanisms of lethality. Exp Opin Ther Targets 2005;9(4):809–24.
[11] Minucci S, Pelicci P. Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer. Nat Rev Cancer 2006;6:38–51.
[12] Masuoka Y, Shin-Ya K, Kim Y-B, Yoshida M, Nagi K, Suzuki K-I. Diheteropeptin, a novel substance with TGF-β activity, produced by a fungus *Diheterospora chlamydosporia*. J Antibiot 2000;53(8):788–92.
[13] Ng H, Bird A. Histone deacetylases: silencers for hire. Trends Biochem Sci 2000;25:121–6.
[14] Bernstein B, Tong T, Schreiber S. Genomewide studies of histone deacetylase function in yeast. Proc Natl Acad Sci (USA) 2000;97(25):13708–13.
[15] Glaser K, Li J, Staver M, Wei R-Q, Albert D, Davidsen S. Role of class I and class II histone deacetylases in carcinoma cells using siRNA. Biochem Biophys Res Commun 2003;310:529–36.
[16] Li J, Staver M, Curtin M, Holms J, Frey R, Edalji R, et al. Expression and functional characterization of recombinant human HDAC1 and HDAC3. Life Sci 2004;74(22):2693–705.
[17] Curtin M, Glaser K. Histone deacetylase inhibitors: the Abbott experience. Curr Med Chem 2003;10:1241–53.
[18] Glaser KB, Li J, Staver MJ, Wei RQ, Albert DH, Davidsen SK. Role of class I and class II histone deacetylases in carcinoma cells using siRNA. Biochem Biophys Res Commun 2003;310(2):529–36.
[19] Zhang C, Richon V, Ni X, Talpur R, Duvic M. Selective induction of apoptosis by histone deacetylase inhibitor SAHA in cutaneous T-cell lymphoma cells: relevance to mechanism of therapeutic action. J Invest Dermatol 2005;125:1045–52.
[20] Piekarz RL, Robey RW, Zhan Z, Kayastha G, Sayah A, Abdeldaim AH, et al. T-cell lymphoma as a model for the use of histone deacetylase inhibitors in cancer therapy: impact of depsipeptide on molecular markers, therapeutic targets, and mechanisms of resistance. Blood 2004;103(12):4636–43.
[21] Duvic M, Talpur R, Ni X, Zhang C, Hazarika P, Kelly C, et al. Phase 2 trial of oral vorinostat (suberoylanilide hydroxamic acid, SAHA) for refractory cutaneous T-cell lymphoma (CTCL). Blood 2007;109(1):31–9.
[22] O'Connor OA, Heaney ML, Schwartz L, Richardson S, Willim R, MacGregor-Cortelli B, et al. Clinical experience with intravenous and oral formulations of the novel histone deacetylase inhibitor suberoylanilide hydroxamic acid in patients with advanced hematologic malignancies. J Clin Oncol 2006;24(1):166–73.
[23] Drummond D, Noble C, Kirpotin D, Guo Z, Scott G, Benz C. Clinical development of histone deacetylase inhibitors as anticancer agents. Annu Rev Pharmacol Toxicol 2005;45:495–528.
[24] Duvic M, Kim Y, Kuzel T, Pacheco T, Foss F, Parker S, et al. Vorinostat (suberoylanilide hydroxamic acid, SAHA) provides clinical benefit to advanced cutaneous T-cell lymphoma patients: updated results of the phase IIb multicenter clinical trial. Blood 2006;108(11) [Abstr# 399].
[25] Konstantinopoulos PA, Vandoros GP, Papavassiliou AG. FK228 (depsipeptide): a HDAC inhibitor with pleiotropic antitumor activities. Cancer Chemother Pharmacol 2006;58(5):711–5.
[26] Furumai R, Matsuyama A, Kobashi N, Lee K-H, Nishiyama M, Nakajima H, et al. FK228(depsipeptide) as a natural prodrug that inhibits class I histone deacetylases. Cancer Res 2002;62:4916–21.
[27] Glaser KB, Li J, Pease LJ, Staver MJ, Marcotte PA, Guo J, et al. Differential protein acetylation induced by novel histone deacetylase inhibitors. Biochem Biophys Res Commun 2004;325(3):683–90.
[28] Tabe Y, Konopleva M, Contractor R, Munsell M, Schober WD, Jin L, et al. Up-regulation of MDR1 and induction of doxorubicin resistance by histone deacetylase inhibitor depsipeptide (FK228) and ATRA in acute promyelocytic leukemia cells. Blood 2006;107(4):1546–54.
[29] Sandor V, Bakke S, Robey RW, Kang MH, Blagosklonny MV, Bender J, et al. Phase I trial of the histone deacetylase inhibitor, depsipeptide (FR901228, NSC 630176), in patients with refractory neoplasms. Clin Cancer Res 2002;8(3):718–28.
[30] Piekarz R, Robey R, Sandor V, Bakke S, Wilson W, Dahmoush L, et al. Inhibitor of histone deacetylation, depsipeptide (FR901228), in the treatment of peripheral and cutaneous T-cell lymphoma: a case report. Blood 2001;98(9):2865–8.
[31] Piekarz R, Frye R, Turner M, Wright J, Allen S, Kirschbaum M, et al. Phase II trial of romidepsin, FK228, in cutaneous and peripheral T-cell lymphoma: clinical activity and molecular markers. Blood 2006;108(11) [Abstr# 2469].
[32] Piekarz R, Fryer A, Wright J, Steinberg S, Liewehr D, Rosing D, et al. Cardiac studies in patients treated with depsipeptide, FK228, in a Phase II trial for T-cell lymphoma. Clin Cancer Res 2006;12(12):3762–73.
[33] Furumai R, Komatsu Y, Nishino N, Khochbin S, Yoshida M, Horinouchi S. Potent histone deacetylase inhibitors built from trichostatin A and cyclic tetrapeptide antibiotics including trapoxin. Proc Natl Acad Sci (USA) 2001;98(1):87–92.
[34] Rosato RR, Almenara JA, Grant S. The histone deacetylase inhibitor MS-275 promotes differentiation or apoptosis in human leukemia cells through a process regulated by generation of reactive oxygen species and induction of p21CIP1/WAF1 1. Cancer Res 2003;63(13):3637–45.

[35] Belien A, De Schepper S, Floren W, Janssens B, Marien A, King P, et al. Real-time gene expression analysis in human xenografts for evaluation of histone deacetylase inhibitors. Mol Cancer Ther 2006;5(9):2317–23.
[36] Ryan QC, Headlee D, Acharya M, Sparreboom A, Trepel JB, Ye J, et al. Phase I and pharmacokinetic study of MS-275, a histone deacetylase inhibitor, in patients with advanced and refractory solid tumors or lymphoma. J Clin Oncol 2005;23(17):3912–22.
[37] Gojo I, Jiemjit A, Trepel JB, Sparreboom A, Figg WD, Rollins S, et al. Phase 1 and pharmacological study of MS-275, a histone deacetylase inhibitor, in adults with refractory and relapsed acute leukemias. Blood 2006.
[38] Grant S. The novel histone deacetylase inhibitor NVP-LAQ824: an addition to the therapeutic armamentarium in leukemia? Leukemia 2004;18(12):1931–3.
[39] Yu X, Guo Z, Marcu M, Neckers L, Nguyen D, Chen G, et al. Modulation of p53, ErbB1, ErbB2, and Raf-1 expression in lung cancer cells by depsipeptide FR901228. J Natl Cancer Inst 2002;94(7):504–13.
[40] Nimmanapalli R, Fuino L, Bali P, Gasparetto M, Glozak M, Tao J, et al. Histone deacetylase inhibitor LAQ824 both lowers expression and promotes proteasomal degradation of Bcr-Abl and induces apoptosis of imatinib mesylate-sensitive and -refractory chronic myelogenous leukemia-blast crisis cells. Cancer Res 2003;63:5126–35.
[41] Fiskus W, Pranpat M, Bali P, Balasis M, Kumaraswamy S, Boyapalle S, et al. Combined effects of novel tyrosine kinase inhibitor AMN107 and histone deaceylase inhibitor LBH589 against Bcr-Abl-expressing human leukemia cells. Blood 2006;108(2):645–52.
[42] Bali P, George P, Cohen P, Tao J, Guo F, Sigua C, et al. Superior activity of the combination of histone deacetylase inhibitor LAQ824 and the FLT-3 kinase inhibitor PKC412 against human acute myelogenous leukemia cells with mutant FLT3. Clin Cancer Res 2004;10:4994–7.
[43] Fiskus W, Herger B, Rao R, Atadja P, Bhalla K. Hydroxamate pan-HDAC inhibitor LBH589 depletes EZH2 and DNMT1 partlty through Hsp90 inhibition and its chaperone association with DNMT1. Blood 2006;108(11) [Abstr# 2233].
[44] Prince M, George D, Johnstone R, McCormack C, Ellis L, Williams-Truax R, et al. LBH589, a novel deacetylase inhibitor (DACi), treatment of patients with cutaneous T-cell lymphoma (CTCL). Skin expression profiles in the first 24 h related to clinical response following therapy. Blood 2006;108(11) [Abstr# 2715].
[45] Plumb JA, Finn PW, Williams RJ, Bandara MJ, Romero MR, Watkins CJ, et al. Pharmacodynamic response and inhibition of growth of human tumor xenografts by the novel histone deacetylase inhibitor PXD101. Mol Cancer Ther 2003;2(8):721–8.
[46] Plumb J, Steele N, Finn P, Brown R. Epigenetic approaches to cancer therapy. Biochem Soc Trans 2004;32(6):1095–7.
[47] Qian X, LaRochelle WJ, Ara G, Wu F, Petersen KD, Thougaard A, et al. Activity of PXD101, a histone deacetylase inhibitor, in preclinical ovarian cancer studies. Mol Cancer Ther 2006;5(8):2086–95.
[48] Tumber A, Collins LS, Petersen KD, Thougaard A, Christiansen SJ, Dejligbjerg M, et al. The histone deacetylase inhibitor PXD101 synergises with 5-fluorouracil to inhibit colon cancer cell growth in vitro and in vivo. Cancer Chemother Pharmacol 2007;60:275–83.
[49] Hose C, Shoemaker R, Monks A. Gene profiling of PXD-101, a novel hydroxamate type inhibitor of histone deacetylase, leads to identification of aurora kinase inhibition. In: AACR-EORTC-NCI molecular targets and cancer therapeutics conference; 2005.
[50] deBono J, Steele N, Vidal L, Plumb J, Attard G, Tjornlund J, et al. A phase 1 pharmacokinetic (PD) and pharmacodynamic (PD) study of the histone deacetylase inhibitor PXD101 in patients with advanced solid tumors. In: AACR-EORTC-NCI molecular targets and cancer therapeutics conference; 2005 [Abstr# C88].
[51] Northfelt D, Ooi C, Gerwien R, Hawthorne T, Clarke A, Jones S, et al. A phase Ib safety and pharmacodynamic study of PXD101 alone and in combination with 5-fluorouracil in patients with advanced solid tumors. In: 18th EORTC-NCI-AACR symposium on molecular targets and cancer therapeutics; 2006 [Abstr# 181].
[52] Sullivan D, Singhal S, Schuster M, Berenson J, Gimsing P, Wisloff F, et al. A phase II study of PXD101 in advanced multiple myeloma. Blood 2006;108(11) [Abstr# 3583].
[53] Lancet J, Nichols G, Assoulin S, Burton M, Mintz M, Green M, et al. A phase 1 study of MGCD0103 given as a twice weekly oral dose in patients with advanced leukemias or myelodysplastic syndromes (MDS). Blood 2006;108(11) [Abstr# 1971.5].
[54] Warrell R, He L-Z, Richon V, Calleja E, Pandolfi P. Therapeutic targeting of transcription in acute promyelocytic leukemia by use of an inhibitor of histone deacetylase. J Natl Cancer Inst 1998;90(21):1621–5.
[55] Zhou D-C, Kim S, Ding W, Schultz C, Warrell R, Gallagher R. Frequent mutations in the ligand-binding domain of PML-RARα after multiple relapses of acute promyelocytic leukemia: analysis for functional relationship to response to all-trans retinoic acid and histone deacetylase inhibitors in vitro and in vivo. Blood 2002;99(4):1356–63.
[56] Yang X, Ferguson AT, Nass SJ, Phillips DL, Butash KA, Wang SM, et al. Transcriptional activation of estrogen receptor alpha in human breast cancer cells by histone deacetylase inhibition. Cancer Res 2000;60:6890–4.
[57] Keen J, Yan L, Mack K. A novel hisotne deacetylase inhibitor, scriptaid, enhances expression of estrogen receptor alpha (ER) in ER-negative human breast cancer cells in combination with 5-aza-2′-deoxycytidine. Breast Cancer Res Treat 2003;81:177–86.
[58] Zhou Q, Atadja P, Davidson N. Hisotne deactylase inhibitor LBH589 reactivates silenced estrogen receptor alpha (ER) gene expression without loss of hypermethylation. Cancer Biol Ther 2007;6(1) [Epub ahead of print].
[59] Sharma D, Saxena N, Davidson N, Vertino P. Restoration of tamoxifen sensitivity in estrogen-receptor negative breast cancer cells: tomoxifen-bound reactivated ER recruits distinctive corepressor complexes. Cancer Res 2006;66(12):6370–8.
[60] Reid G, Metivier R, Lin CY, Denger S, Ibberson D, Ivacevic T, et al. Multiple mechanisms induce transcriptional silencing of a subset of genes, including oestrogen receptor alpha, in response to deacetylase inhibition by valproic acid and trichostatin A. Oncogene 2005;24(31):4894–907.
[61] Munshi A, Tanaka T, Hobbs M, Tucker S, Richon V, Meyn R. Vorinostat a histone deacetylase inhibitor, enhances the response of human tumor cells to ionizing radiation through prolongation of gama-H2AX foci. Molec Cancer Ther 2006;5(8):1967–74.
[62] Kao G, McKenna W, Guenther M, Muschel R, Lazar M, Yen T. Histone deacetylase 4 interacts with 53BP1 to mediate the DNA damage response. J Cell Biol 2003;160(7):1017–27.
[63] Longley DB, Harkin DP, Johnston PG. 5-Fluorouracil: mechanisms of action and clinical strategies. Nat Rev Cancer 2003;3(5):330–8.
[64] Glaser K, Staver M, Waring J, Stender J, Ulrich R, Davidsen S. Gene expression profiling of multiple histone deacetylase (HDAC) inhibitors: defining a common gene set produced by HDAC inhibition in T24 and MDA carcinoma cell lines. Mol Cancer Ther 2003;2:151–63.
[65] Ocker M, Alajati A, Ganslmayer M, Zopf S, Luders M, Neureiter D, et al. The histone-deacetylase inhibitor SAHA

potentiates proapoptotic effects of 5-fluorouracil and irinotecan in hepatoma cells. J Cancer Res Clin Oncol 2005;131:385–94.
[66] Zhang X, Yashiro M, Ren J, Hirakawa K. Histone deacetylase inhbitor, trichostatin A, increases the chemosensitivity of anticancer drugs in gastric cancer cell lines. Oncol Rep 2006;16:563–8.
[67] Berg RW, Ferguso PJ, DeMoor JM, Vincen MD, Koropatnick J. The means to an end of tumor cell resistance to chemotherapeutic drugs targeting thymidylate synthase: shoot the messenger. Curr Drug Targets 2002;3(4):297–309.
[68] Matsuoka K, Tsukuda K, Suda M, Kobayashi K, Ota T, Okita A, et al. The transfection of thymidylate synthase antisense suppresses oncogenic properties of a human colon cancer cell line and augments the antitumor effect of fluorouracil. Int J Oncol 2004;24(1):217–22.
[69] Lee J-H, Park J-H, Jung Y, Kim J-H, Jong H-S, Kim T-Y, et al. Histone deactylase inhibitor enhances 5-fluorouracil cytotoxicity b down-regulating thymidylate synthase in human cancer cells. Mol Cancer Ther 2006;5(12):3085–95.
[70] Fiskus W, Pranpat M, Balasis M, Bali P, Estrella V, Kumaraswamy S, et al. Cotreatment with Vorinostat (suberoylanilide hydroxamic acid) enhances activity of Dasatinib (BMS-354825) against Imatinib mesylate-sensitive and Imatinib mesylate-resistant chronic myelogenous leukemia cells. Clin Cancer Res 2006;12(19):5869–78.
[71] Strahl BD, Allis CD. The language of covalent histone modifications. Nature 2000;403(6765):41–5.
[72] Turner BM. Histone acetylation and an epigenetic code. Bioessays 2000;22(9):836–45.
[73] Turner B. Reading signals on the nucleosome with a new nomenclature for modified histones. Nat Struct Molec Biol 2005;12(2):110–2.
[74] Turner BM. Defining an epigenetic code. Nat Cell Biol 2007;9(1):2–6.
[75] Esteller M. Epigenetics provides a new geneation of oncogenes and tumour-suppressor genes. Br J Cancer 2006;94:179–83.
[76] Huang J, Perez-Burgos L, Placek B, Sengupta R, Richter M, Dorsey J, et al. Repression of p53 activity by Smyd2-mediated methylation. Nature 2006;444:629–32.
[77] Shi Y, Whetstine JR. Dynamic regulation of histone lysine methylation by demethylases. Mol Cell 2007;25(1):1–14.
[78] Lehrman G, Hogue IB, Palmer S, Jennings C, Spina CA, Wiegand A, et al. Depletion of latent HIV-1 infection in vivo: a proof-of-concept study. Lancet 2005;366(9485):549–55.
[79] Ylisastigui L, Archin NM, Lehrman G, Bosch RJ, Margolis DM. Coaxing HIV-1 from resting CD4 T cells: histone deacetylase inhibition allows latent viral expression. AIDS 2004;18(8):1101–8.
[80] Brogdon J, Xu Y, Szabo S, An S, Buxton F, Cohen D, et al. Histone deacetylase activities are required for innate immune cell control of Th1 but not Th2 effector cell function. Blood 2007;109(3):1123–30.
[81] Inoue K, Kobayashi M, Yano K, Miura M, Izumi A, Mataki C, et al. Histone deacetylase inhibitor reduces monocyte adhesion to endothelium through the suppression of vascular cell adhesion molecule-1 expression. Arterioscler Thromb Vasc Biol 2006;26(12):2652–9.
[82] Faraco G, Pancani T, Formentini L, Mascagni P, Fossati G, Leoni F, et al. Pharmacological inhibition of histone deacetylases (HDACs) by suberoylanilide hydroxamic acid (SAHA) specifically alters gene expression and reduces ischemic injury in the mouse brain. Molec Phramacol 2006;70(6):1876–84.
[83] Butler R, Bates GP. Histone deacetylase inhibitors as therapeutics for polyglutamine disorders. Nat Rev Neurosci 2006;7(10):784–96.
[84] Sadri-Vakili G, Cha JH. Histone deacetylase inhibitors: a novel therapeutic approach to Huntington's disease (complex mechanism of neuronal death). Curr Alzheimer Res 2006;3(4):403–8.
[85] Haggarty S, Koeller K, Wong J, Grozinger C, Schreiber S. Domain-selective small-molecule inhibitor of histone deacetylase 6 (HDAC6)-mediated tubulin deacetylation. Proc Natl Acad Sci (USA) 2003;100(8):4389–94.

Pharmaceuticals **2010**, *3*, 2022-2044; doi:10.3390/ph3072022

OPEN ACCESS
pharmaceuticals
ISSN 1424-8247
www.mdpi.com/journal/pharmaceuticals

Review

Demethylating Agents in the Treatment of Cancer

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Received: 4 May 2010; in revised form: 22 June 2010 / Accepted: 29 June 2010 / Published: 2 July 2010

Abstract: Gene silencing resulting from aberrant DNA methylation can lead to tumorigenesis. Therefore, drugs that inhibit or interfere with DNA methylation have been used to reactivate and induce silenced gene re-expression in malignancies. Two demethylating agents, azacitidine and decitabine, are approved for the treatment of myelodysplastic syndromes (MDS) by the U.S. Food and Drug Administration (FDA), and are now considered the standard of care in MDS. In this review, we discuss clinical data, including clinical benefits and toxicities, which led to the approval of azacitidine and decitabine. We also summarize findings from clinical trials that used these two demethylating agents in the treatment of solid tumors. Lastly, we discuss some limitations in the use of azacitidine and decitabine in cancer therapy.

Keywords: azacitidine; cancer; decitabine; epigenetics; methylation

1. Introduction

Epigenetics was one of the most popular focuses of cancer research in the last decade. While genetic aberrations such as mutation, deletion and translocation form much of the basis for what we know about acquired and spontaneously developed disease states, including cancer, epigenetics, defined as heritable changes in gene expression that do not involve alterations in DNA sequence, has indeed provided another level of gene expression regulation still present after multiple cell divisions whereby direct DNA modification is not required to promote the development and progression of a tumor. Instead, epigenetic events such as hypermethylation of gene promoter regions (blocking

binding sites of transcription factors) [1], global DNA hypomethylation, histone tail modifications (including combinations of methylation and acetylation, among others), small RNA (including microRNA) expression, and higher order chromatin folding all act and interact to manage the expression of individual and collections of genes, responsible for either the maintenance of cellular homeostasis and 'correct' activity or, when deregulated, inducing the cell to evade apoptosis, proliferate and potentially invade tissues and metastasize. While many of these processes are not novel, more recent studies tying epigenetic mechanisms to the expression of tumor suppressor genes and oncogenes [1], the subsequent activities of relevant signaling pathways, as well as reporting them as readily detectable independent diagnostic and prognostic tumor biomarkers [2–5], have propelled this field of research to the forefront of clinical cancer therapeutics. Furthermore, unlike genetic mutations, epigenetic aberrations are inherently reversible, thus making the use of targeted therapies against them very attractive. Over the past decade, various drugs have been developed to target such processes, many with promising results in clinical trials, while some older compounds have become better managed through more calculated dosing regimens to achieve their projected potential as effective antineoplastic agents, namely the hypomethylation-inducing cytidine analogs 5-azacytidine (azacitidine, 5-aza-CR; Vidaza®, Celgene Corp., Summit, NJ, USA) and 5-aza-2'-deoxycytidine (decitabine, 5-aza-CdR; Dacogen®, SuperGen, Inc., Dublin, CA, USA).

In this review, we will discuss data from clinical trials using azacitidine and decitabine in hematologic and solid malignancies as well as discuss their therapeutic limitations. In particular, we will focus on the clinical evolution of azacitidine and decitabine and their approval by the U.S. Food and Drug Administration (FDA) for the treatment of myelodysplastic syndromes (MDS).

2. DNA Methylation

Of all known mammalian epigenetic modifications, DNA methylation is likely the most widely and intensively studied. As a second tier of gene expression regulation along with chromatin folding, gene promoter methylation provides a physical blockage of the DNA binding site for transcription factors while further inhibiting transcription through the recruitment of chromatin modifying proteins via methyl-CpG binding proteins (MBPs) [6]. Transcriptionally inactive heterochromatin, tightly compacted, typically harbors hypermethylated DNA while active euchromatin is conversely unmethylated [7]. DNA methylation is directed by DNA methyltransferases (DNMT1, DNMT3A, DNMT3B) which transfer a methyl group from *S*-adenosyl-l-methionine to the cytosine of a CpG (cytosine-phosphate-guanine) dinucleotide (adjacent within a single DNA strand) immediately following replication. Since a CpG on one strand of DNA will pair with a CpG on the other, there exists the possibility for either completely unmethylated (neither CpG), hemimethylated (one CpG) or fully methylated (both CpG:CpG) sites. DNMT1, the most abundant DNMT in mammals, is an important mediator of *de novo* and maintenance methylation of unmethylated and hemimethylated sites, respectively, but preferentially binds hemimethylated sites to restore full CpG pair methylation after replication has resulted in one unmethylated daughter strand [8,9]. When DNMT1 levels are reduced, as is the case following azacitidine or decitabine treatment, daughter strands are less likely to undergo such maintenance to restore full methylation; thus, with each replication, CpG pairs become unmethylated, their promoter regions now more accessible to transcription factors.

In the vertebrate genome, ~37% of CpGs locate in the 5'-regulatory promoter regions of 60–70% of genes in CpG 'islands', regions of the genome with a high frequency of CpGs [10,11]. The reason for such a discrepant localization may be explained by CpG developmental character. Methylated CpGs (meCpGs) have an increased propensity for transition mutation to TpG, and since most CpGs are methylated in normal human cells, the relatively high frequency of CpGs in promoter regions has been attributed to their evolutionarily conserved unmethylated status [3], typically maintained during development and differentiation [12]. However, promoter hypermethylation during development is also common and well described [13–15], resulting in long-term gene silencing, e.g., X-chromosome inactivation [16] and gene imprinting [17].

When methylation machinery becomes deregulated, as through the increased expression/activity of DNMTs, reduced expression/activity of their negative regulators, reactive oxygen species (ROS) formation as due to chronic inflammation, and/or the presence of carcinogens, resulting promoter hypermethylation may lead to a variety of pro-tumorigenic outcomes. For example, when methylated, expression of tumor suppressor genes, such as the retinoblastoma gene (*RB1*), E-cadherin (*CDH1*), and the DNA repair gene O6-methylguanine-DNA methyltransferase (*MGMT*), is reduced, leading to events such as an increased frequency of mutation and microsatellite instability [18,19]. Currently, methylated *MGMT* is being utilized as a valuable marker of chemosensitivity to alkylating agents in patients with gliomas, further emphasizing the translational potential of such epigenetic mechanisms into the realm of cancer prognostics [19–22]. Of note, CpG islands within the 3' regions of genes as well as intergenic islands may be hypermethylated in cancer [23,24], while an increased expression of some 3' meCpG genes has also been described [23]. Such evidence implies for the multifaceted influence of even singular epigenetic processes over gene expression. It is therefore incumbent upon us to continue to study and learn to manipulate such an important epigenetic event for the purpose of inducing silenced tumor suppressor gene expression [25], increasing the possibilities for appropriate apoptotic response and reversing tumor progression.

Differences in methylation patterns of normal and tumor cells were first described around 30 years ago, in particular that tumor cells displayed a global hypomethylation relative to normal cells of the same type [26–29]. Advancing the early use of Southern blotting and restriction enzymes for methylation profiling, techniques such as bisulfite sequencing with methylation-specific PCR (MSP), array-based detection, and immunoprecipitation of meCpGs have been employed to define cancers based on global DNA methylation status [30]. Indeed, many normal and cancerous tissues have been profiled using these methods, exhibiting commonalities of global hypomethylation and promoter hypermethylation of many recognized tumor suppressor genes [31–33] and miRNA [34,35] as well as displaying tissue-specific methylation patterns [31,36]. Furthermore, the interplay of CpG methylation with histone [37,38] and miRNA [35,39–42] gene regulatory mechanisms makes DNA methylation an important focus of translational cancer research. Given that normal cells typically display unmethylated promoter CpGs, targeted therapeutics against methylation includes an inherent specificity for tumor cells.

3. Development and Utility of Azacitidine and Decitabine for Cancer Therapy

3.1. Mechanistics

Azacitidine and its deoxy derivative decitabine are cytidine analogs that contain a nitrogen in place of the 5-carbon of the pyrimidine ring. Each enters the cell via nucleoside transporters, such as the confirmed human concentrative nucleoside transporter 1 (hCNT1) and equilibrative nucleoside transporter 1 (hENT1) [43-45]. Once inside the cell they undergo three steps of phosphorylation to achieve their active forms. The initial rate-limiting monophosphorylation by uridine-cytidine kinase (UCK; azacitidine) and deoxycytidine kinase (dCK; decitabine) is followed by phosphorylation to their diphosphate forms. Reduction of ~10% of azacitidine diphosphate to its deoxy form, decitabine diphosphate, by ribonucleotide reductase allows both drugs to target DNA, albeit with different potencies, as decitabine triphosphate can incorporate into DNA inducing CpG demethylation and chromosomal instability, while the remaining ~90% of azacitidine triphosphate incorporates into messenger and transfer RNA, inhibiting protein synthesis. Importantly, since decitabine phosphorylation only proceeds toward targeting DNA, it exhibits a more potent inhibition of DNA methylation and is at least 10 times more cytotoxic than azacitidine [46–48]. Cytidine deaminase acts to deaminate and open up the azanucleotide ring structure leading to metabolism of the nucleotide [49] with primary excretion via the kidneys; however, van Groeningen *et al.* reported on the significant role of metabolic breakdown of decitabine in the body as they observed high drug clearance with less than 1% of the administered dose excreted in urine in a phase I trial of 21 patients with advanced solid tumors [50].

DNMT1 irreversibly binds DNA-incorporated decitabine triphosphate, reducing available DNMT1 in the nucleus through sequestration and its subsequent degradation, thus passively inhibiting DNA methylation [46,51–53]. Clinically, DNMT1 protein levels may be assayed for to assess incorporation of the azanucleoside [54]. Furthermore, the binding of DNMT1, typically found in multi-unit enzyme complexes with chromatin remodelers such as histone deacetylases (HDAC) [55,56], to an azanucleotide may induce structural and functional changes in the complex, reducing chromatin folding and further promoting gene expression.

The pharmacologic effects of high-dose azanucleosides may be due in large part to the cytotoxicity of azanucleotide:DNMT adducts formed in DNA, potentially masking the importance of demethylation via clonal expansion of resistant cells [46,57]. Lower doses instead induce adduct degradation without inhibition of DNA synthesis, thus allowing for optimal extended treatment durations [58]. As demethylation induces the re-expression of silenced genes from various pathways, e.g. apoptosis, differentiation, angiogenesis, senescence, *etc.*, many of which have tumor and patient-specific activity, we must also keep in mind the inherent difficulties associated with such non-specific targeting with demethylating agents in predicting patient response.

3.2. Development and Preliminary Data

Piskala and Sorm first described their synthesized nucleoside analogs azacitidine and decitabine as highly active cytotoxic agents against lymphatic leukemia in mice and cell lines, able to induce chromosomal breakage [46,59], nearly 45 years ago [60,61]. Each was further shown to induce

differentiation of multipotent mouse embryonic 10T1/2 cells [62,63], later reported to be the result of their inhibition of DNA methylation [64,65]. Hematopoietic cancers have a particularly high degree of aberrant methylation [66]. Further supporting the roles of azanucleosides in differentiation, an increase of fetal hemoglobin was observed after treatment with azacitidine in patients with β-thalassemia [67] and sickle cell anemia [68], and monoblastic and myeloblastic leukemia cells were induced to differentiate upon treatment with decitabine [69]. Multiple treatment regimens of decitabine in L1210 leukemic mouse xeonografts showed promising increases in life span with a significant lag in tumor cell proliferation [70], and greater than 70% inhibition of DNA methylation was observed in blood cells from patients with lymphatic and myeloid leukemias [48]. Early data from *in vivo* solid tumor models showed little effect [61]; however, with the continuous identification of methylated tumor suppressors in solid tumors came a heightened interest in optimizing drug delivery systems and dosing and reducing toxicity for future therapy.

1971 saw an application by the National Cancer Institutes to the FDA for Investigational New Drug status, indicating azacitidine as an antineoplastic agent for the treatment of multiple cancers. Clinical trials had begun in Europe in 1967 and in the U.S. in 1970 including more than 800 evaluable patients with such conditions as acute and chronic myelogenous leukemia (AML, CML), acute lymphocytic leukemia (ALL), and breast, colorectal, lung, and melanoma solid tumors [61]. Azacitidine was quite effective in treating AML, in particular for patients with an initial or developed resistance to common treatment regimens of that time and/or with relapse, thus providing a valuable niche for azacitidine. Of 200 patients, 41 (20%) exhibited complete disease remission with 32 (16%) partial remissions following treatment with azacitidine, the majority of responding patients having been refractory to conventional chemotherapeutics. Administration in these studies ranged from 15 doses of 60 mg/m^2 every eight hours to five daily doses of 500 mg/m^2 to a single infusion of 750 mg/m^2. However, responses were either low or non-existent in CML, ALL and multiple myeloma. Data from solid tumor trials either showed poor response or were inadequate for deriving any significant conclusion. Toxicity was particularly disconcerting as 73% of all 745 patients reported to the Investigational Drug Branch exhibited nausea and vomiting within three hours of each series of intravenous (i.v.) injections, and 53% of patients exhibited diarrhea. Sporadic yet occasionally severe myalgia was reported while incidences of dose-limiting leukopenia and thrombocytopenia were 34% and 17%, respectively. Overall, few deaths were associated with treatment, most (N = 4) reported in patients with unhealthy livers [71]. Importantly, an escalation toward maximum tolerated dose, a common protocol for early trials, may not be the best way to observe such a drug, as azacitidine is most efficacious at low doses, inhibiting DNA synthesis at high doses [58,61,72]. A more relaxed regimen of 75 mg/m^2d for seven days every 28 days would later prove more effective and less toxic in a subset of cancer patients.

The FDA rejected this application for azacitidine due at least in part to an unacceptable level of toxicity relative to observed antitumor effectiveness; however, these collective data served to promote intrigue regarding the actions and development of epigenetic therapies for cancer. Four decades would ultimately pass before the first such drug gained approval for clinical use, carrying with it an immediate impact in the treatment of hematologic malignancies, in particular MDS.

3.3. FDA Approval for Myelodysplastic Syndromes

3.3.1. Myelodysplastic Syndromes

MDS are a collection of hematopoietic stem cell disorders characterized by bone marrow dysplasia and peripheral blood cytopenia, sometimes referred to as preleukemias due to their tendency to transform into AML [73]. Five subtypes of MDS are designated under the original French-American-British (FAB) classification system, including: (1) refractory anemia (RA); RAs with (2) ring sideroblasts (RARS; high iron content in red blood cells); (3) excess blasts in bone marrow (RAEB) and a (4) high excess of blasts in transformation to AML (RAEB-T; low level of circulating white blood cells and platelets); as well as (5) chronic myelomonocytic leukemia (CMML; high level of circulating monocytes, variable red and white blood cells and platelet levels) [74]. The World Health Organization (WHO), the International Working Group (IWG), sponsored by the National Cancer Institutes (NCI), and International Prognostic Scoring System (IPSS), the most common prognostic scoring system for MDS, have since made important adjustments to the somewhat dynamic classification and response criteria of MDS, with the notable exclusion of CMML [75–79].

The cellular heterogeneity of MDS provides a valuable prognostic tool for a historically difficult cancer to treat. Bone marrow mononuclear cells from MDS patients exhibited gene expression signatures similar to AML, MDS and non-leukemic cells in a 1:2:1 relationship, respectively, while AML-like cells were in abundance (68%) in WHO-designated RAEB-2 (high-risk, transforming) marrow [80]. Most high-risk, RAEB malignancies result in patient mortality within one year, nearly half transforming into AML [81]. Unfortunately, effective treatment of these patients has been problematic in the past as high-dose cytotoxic agents have produced generally poor results. Importantly, as most patients diagnosed with an MDS are over 70 years old [82], allogeneic hematopoietic stem-cell transplantation (SCT), which produces the best long-term disease remission but also has a high rate of treatment-related death (39% after one year) and significant graft-*versus*-host disease [83], is not warranted except in cases of advanced disease [84], encompassing only about 5% of all MDS patients [85]. Supportive care, sometimes including blood transfusion, administration of hematopoietic growth factors, or chemotherapy, had generally been the standard of care for many low- and high-risk MDS patients prior to the availability of azacitidine and decitabine with effective dosing regimens. Notably, recent studies indicate the feasibility of pre-treating high-risk MDS/AML patients with decitabine prior to SCT, citing no unexpected toxicities [86,87].

3.3.2. Azacitidine

In a second Investigational New Drug application, this time for the specific use of azacitidine in patients with MDS, Pharmion Corp. (acquired by Celgene Corp. in 2008) submitted data from three open-label, multicenter trials of the Cancer and Leukemia Group B (CALGB) [81,85,88,89]. After initial data was generated under CALGB and original FAB criteria, a reanalysis using updated WHO and IWG classification and response criteria, respectively, was completed [90]. CALGB 9221 [85] was the lone controlled trial testing subcutaneous (s.c.) injections of azacitidine in patients with all five types of MDS against those solely under supportive care, while CALGB 8921 [88] and 8421 [89] tested s.c. and i.v. injections, respectively, without a control arm. Marcucci *et al.* have since reported a

two-fold higher beta (substance elimination) half-life for s.c. injections of azacitidine (41 min) over i.v. (20 min) in patients with MDS [91]. Dosing for all trials was 75 mg/m^{2}d given for seven consecutive days every 28 days. All staging and response criteria were identical for the three trials. Patients with an adjudicated AML diagnosis at baseline were not included in the analyses of transformation to AML and response rate. Complete response (CR) is defined as complete normalization of bone marrow and peripheral blood counts; partial response (PR) as > or = 50% restoration of the deficit from normal of all three peripheral blood cell lines, elimination of transfusion requirements, and a decrease in percentage bone marrow blasts by > or = 50% from pre-study values; hematologic improvement (HI) as > or = 50% restoration in the deficit from normal of one or more peripheral blood cell lines and/or a > or = 50% decrease in transfusion requirements; and total measurable response as CR + PR + HI.

An early CALGB phase II trial [89] included 43 evaluable hospitalized patients with high-risk RAEB or RAEB-T. Under original CALGB/FAB criteria, complete response was seen in five (12%) patients, partial response in 11 (25%), and hematologic improvement in five (12%) patients for a total measurable response in 21 (49%) patients. Median survival for all patients was 13.3 months with median duration of response for those with complete and partial response at 14.7 months. Median time to response was three cycles. Toxicities included nausea and vomiting (63% incidence) and dose-limiting myelosuppression (33%).

The second phase II trial, CALGB 8921 [88,92], exhibited similar response rates, duration and survival to the first, albeit via an s.c. daily bolus injection of an evaluable 70 patients of RAEB, RAEB-T and CMMT subtypes. Complete responses were noted for 12 (17%) patients and hematologic improvement for 16 (23%), while toxicity, save for an occasional skin reaction at site of injection, and median time to response were similar to the other trials.

In the multicenter (26 academic centers, 30 affiliates), randomized, open-label and controlled CALGB 9221 trial [85,92], 191 patients encompassing each MDS subtype, including 20 AML, were stratified according to FAB classification and randomly separated into azacitidine treatment arm (N = 99) and observational, supportive therapy arm (N = 92), additionally matched across arms according to patient physical (e.g., age, gender, race, weight), disease (e.g., FAB subtype, cytogenic analysis, IPSS classification, time from diagnosis to study entry), and treatment history characteristics. Patients were allowed to cross over from the observational to treatment arm after a minimum of four months if disease progressed, whereby they were treated and studied identically to those initially randomized to the treatment arm. Four cycles after azacitidine treatment, patient bone marrow biopsies were analyzed. Patients with complete response continued on treatment for an additional three cycles, while those with partial response or hematologic improvement continued until achieving a complete response or relapse.

Of 99 patients in the treatment arm, seven (7%) exhibited CR; 16 (16%), PR; and 37 (37%), HI, for a total measurable response of 60 (60%), each a statistically significant response as compared to observation. Alternatively, only five of 92 (5%) patients in the supportive care arm showed any response (HI). Median time to initial response and best response was 64 days (cycle 3) and 93 days (cycle 4), respectively, as median duration of response for those with CR, PR or HI was 15 months. Patients who either crossed over from the observational to the treatment arm after six months or did not cross over at all exhibited a significantly lower median overall survival (11 months) than those initially randomized to the azacitidine arm (18 months). Furthermore, of the 49 who did in fact cross

over at some point, 23 (47%) responded to treatment with five (10%) patients achieving CR; two (4%), PR; and 16 (33%), HI. Median overall survival was 20 months for all patients in the azacitidine arm *versus* 14 months for those who remained under supportive care. Time to progression to either AML or death was significantly (P = 0.04) longer (19 months) than for that of the observational arm (eight months). During the first six months of the trial, 3% of patients in the azacitidine arm transformed into AML, while 24% from observation transformed (P < 0.0001).

Quality of life was also shown to improve significantly for those treated with azacitidine, including those who crossed over [93], compared with the observational arm, which either remained stable or worsened with time. Toxicities associated with azacitidine treatment include transient cytopenias, grade 3 or 4 leukopenia (43% incidence), granulocytopenia (58%), and thrombocytopenia (52%) with bleeding as well as infection (20%), nausea and vomiting (4%), and one potentially treatment-related death. Other common events include myalgia, weakness, fatigue, rash, erythema, limb pain, neutropenia, pneumonia, coughing, dyspnea, constipation, and fever. The frequency of such events decreased after the first two cycles of therapy. Fifty patients were discontinued from the trials for showing no response after four cycles of treatment. Overall, azacitidine is a significantly more effective antineoplastic agent than supportive care for MDS, improving overall survival, bone marrow function and quality of life while reducing and delaying MDS transformation into AML.

The application of updated WHO, IWG and IPSS criteria to data from the CALGB trials has served to validate these findings, the major change being a more precise definition of RAEB and RAEB-T, evident by the increased number of RAEB and RAEB-T patients re-characterized as AML in each trial [90]. Overall response rates indeed remained consistent. On May 19, 2004, the FDA approved azacitidine (Vidaza®) as an injectable suspension for the treatment of all five FAB subtypes of MDS (including RARS, if accompanied by neutropenia or thrombocytopenia or requiring transfusions) [92].

3.3.3. Decitabine

The efficacy of decitabine infusion in pediatric and adult leukemia patients has been described, resulting in 20% and 33% complete response rates for those with ALL and AML, respectively [94]. Phase II trials of decitabine (45 mg/m^{2}d for three days every six weeks) as treatment for MDS in older patients resulted in complete response rates of 20-28% and overall response rates of 42–54% [95,96].

These data led to the initiation of a multicenter, open-label, randomized, and controlled phase III trial for decitabine *versus* supportive care in 170 patients with all five FAB sub-types of MDS and IPSS-designated intermediate-1, intermediate-2 and high-risk, 70% of all patients being of intermediate-2 or high-risk IPSS type [97]. Patients were randomized into two groups, decitabine plus best supportive care (N = 89) and solely best supportive care (N = 82). The dosing regimen was continuous i.v. infusion of 15 mg/m^2 over three hours every eight hours for three days, repeating every six weeks.

Durable response seen for patients in the decitabine arm was 17% (15 of 89 patients, 9% CR + 8% PR) with 13% HI, and 0% in the control, supportive care arm (P<0.001). For the decitabine arm, median time to response was 3.3 months and median duration of response was 10.3 months. Patients in the decitabine arm also tended to have a longer median time to develop AML or death than patients in the supportive care group (all patients, 12.1 *vs.* 7.8 months, P = 0.16), and statistically significant

values were seen for intermediate-2/high-risk (P = 0.03) and *de novo* (P = 0.04) disease groups. Two multicenter, open-label, single-arm trials of decitabine (15 mg/m^2 continuous infusion for four hours every eight hours over three days, repeating every six weeks) for 164 total patients with MDS of any FAB sub-type were further initiated, resulting in overall response rates of 26% (N = 66) and 24% (N = 98) [98]. Nausea, vomiting, constipation, diarrhea, fever, hyperglycemia, back pain, coughing, headache, insomnia, rash, and petechiae were among the more common side effects, while the primary toxicity of decitabine was myelosuppression.

On May 2, 2006, the FDA approved decitabine (Dacogen®) as an injectable suspension for the treatment of all original FAB subtypes of MDS as well as intermediate-1, intermediate-2, and high-risk groups of the IPSS. Recently, March 11, 2010 saw the FDA approval of a five-day out-patient dosing regimen for Dacogen® for injection. The regimen consists of a 20 mg/m^2 continuous i.v. infusion over one hour repeated daily over a five day cycle, repeating every four weeks. This regimen aims to improve upon the previous in-patient dosing of 15 mg/m^2 i.v. over three hours repeated every eight hours for three days per cycle, repeating every six weeks. Three multicenter, open-label, single-arm studies evaluated the efficacy of this decitabine treatment regimen for patients with MDS of any FAB sub-type [99]. Based on IWG 2000 criteria, the overall response rate was 16% (15% CR, 1% PR) with a median time to response of 162 days and median duration of response of 443 days. Hematologic toxicities, including neutropenia (37%), thrombocytopenia (24%) and anemia (22%), and infections were the most prevalent toxicities, accounting for most dose delays and patient discontinuation, possibly contributing at least in part to eight infection- and/or bleeding-associated deaths. Fatigue, nausea, coughing, constipation, and diarrhea were listed among common adverse events.

3.4. Azacitidine, Decitabine and Chemotherapy for High-Risk MDS Patients

Current National Comprehensive Cancer Network guidelines recommend the use of hypomethylating agents for IPSS-classified high-risk MDS patients who are not candidates for conventional chemotherapy.

In a recent multicenter, randomized trial for patients of all prognostic sub-groups of high-risk MDS, azacitidine (75 mg/m^{2}d for seven days every 28 days) nearly doubled their overall survival rate (50.8% *vs.* 26.2%) past that of conventional therapy, including best supportive care, low-dose cytarabine or intensive chemotherapy [100]. The study, carried out by the International Vidaza® High-Risk MDS Survival Study Group, enrolled 358 patients at 79 sites in 15 countries. Azacitidine induced more serious hematologic side effects than the best supportive care, but fewer than chemotherapy, and it lowered the risk of patient infection by one-third compared with conventional care. However, there was no significant advantage over chemotherapy, potentially due to the few available patients suitable for chemotherapy. Response rates of 17% complete and 12% partial indicated little benefit over chemotherapy or combination treatment [101]. Data from a meta-analysis of three trials including over 900 MDS patients testing azacitidine and decitabine against these same conventional therapies suggests instead a prolonged overall survival and time to AML transformation or death, improved CR, PR, HI, and overall response after treatment [102]. A phase III clinical trial has recently been initiated to demonstrate the "superiority" of decitabine over azacitidine for the treatment of these intermediate- or high-risk MDS patients (http://www.clinicaltrials.gov/, NCT01011283).

4. Azacitidine and Decitabine in the Treatment of Solid Tumors

Demethylating agents seem to be more effective in patients with hematologic malignancies rather than solid tumors and effective at much lower doses; however, mountains of basic research data tell us that methylation is a common driving force behind many facets of tumorigenesis, growth and metastasis [103]. Indeed, a PubMed search for "DNA methylation cancer" returns more than 12,000 articles. While we can efficiently utilize azanucleosides for the manipulation of numerous types of solid tumor cells *in vitro*, their translation to therapeutics has been somewhat of a mystery. With all that we now know about epigenetic regulation of cancer progression via DNA methylation, effective treatment of solid malignancies with demethylating agents in the clinical setting seems to be just around the corner.

The use of both azacitidine and decitabine in clinical trials for solid tumors has been and is now only occasionally attempted, though the main focus of these drugs for years has undoubtedly regarded hematological malignancies. Solid tumor trials have included gastrointestinal, lung, ovarian, prostate, breast, and head and neck cancers, melanoma and malignant mesothelioma [104–115]. While some groups have shown strong response (seven of 11 breast cancer patients (63%) responded to i.v. administration of 300–700 mg/m^2 over an eight day period), others with larger patient cohorts give little to no response at all. Given the recent success of the azanucleosides in treating MDS, AML and other hematologic cancers, further study into the *in vivo* action of demethylating agents in solid tumor systems is highly warranted.

In a small clinical trial, Momparler *et al.* treated 15 patients with stage IV non-small-cell lung carcinoma with a decitabine regimen of 200–600 mg/m^2 over eight hours [109]. Median survival for nine evaluable patients was 6.7 months, with three patients surviving at least 15 months. Hellebrekers *et al.* have described the *in vitro* and *in vivo* anti-angiostatic abilities of decitabine, potentially adding another important mechanism for its anti-neoplastic activity [116]. A 168 hours continuous i.v. infusion of decitabine in 10 patients with refractory solid tumor has been described as well tolerated [117]. Promoter-specific and global DNA methylation levels were significantly decreased by day 14, expression change verified by quantitative RT-PCR, with reversion back to baseline 28 to 35 days after treatment had commenced, indicating the transient effect of decitabine on *in vivo* methylation. In 1983, a phase I trial and pharmacokinetic study of decitabine in 21 patients with advanced solid tumors resulted in one partial response [50]. Nineteen patients with metastatic solid tumors were given a dose between 20 and 40 mg/m^2 via continuous i.v. infusion for 72 hours. After seven days, some gene demethylation was identified, but there were no objective responses to the treatment [118].

Beyrouthy *et al.* describe the hyper-sensitization of pluripotent embroyonal carcinoma (EC) cells to low levels (IC_{50}, 5–25 nmol/L) of decitabine in the presence of DNMT3B [119]. Additionally, cisplatin-resistant EC cells, the 'stem cells' of testicular germ cell tumors, may be re-sensitized to cisplatin toxicity after pre-treatment with decitabine in the presence of DNMT3B.

Another recent study found that azacitidine therapy significantly reduced median prostate-specific antigen (PSA) doubling time, a sign of improved long-term patient outcome, in men with chemonaïve castration-resistant prostate cancer, correlating with decreased plasma DNA long interspersed nuclear

element 1 (LINE-1) methylation levels [120]. The older majority of prostate cancer patients may benefit from such therapy as only minor toxicities were reported.

We have recently initiated a single-center phase I/II clinical trial testing combination azacitidine with the chemotherapeutic, nanoparticle albumin-bound paclitaxel (nab-paclitaxel; Abraxane®, Abraxis Bioscience, Los Angeles, CA), for the treatment of patients with advanced or metastatic solid tumors and breast cancer is currently recruiting participants (http://www.clinicaltrials.gov/, NCT00748553). The effective antineoplastic ability of nab-paclitaxel within some tumors is thought to result from its accumulation at the tumor site via the actions of SPARC, secreted protein acidic and rich in cysteine, a secreted glycoprotein overexpressed in a variety of tumors [121]. The presence of bound albumin allows for receptor-mediated transcytosis of nab-paclitaxel across the endothelium. Upon entering the tumor interstitium, SPARC then binds and sequesters albumin, releasing paclitaxel inside the tumor. Low SPARC expression has been described in colon, lung, ovarian, pancreatic, and cervical cancer cell lines, correlating with promoter hypermethylation in tested cases [122–127]. Use of a demethylating agent has been shown to induce SPARC expression in some of these cell types [123,126]. In a recent study, SPARC expression was able to inhibit breast cancer metastasis [128]. Our clinical trial intends to up-regulate SPARC with an initial treatment of azacitidine to increase the efficacy of nab-paclitaxel and joint effectiveness of both drugs in solid tumors.

5. Limitations of Azacitidine and Decitabine

An important limitation for such demethylating agents is their inherent need for actively dividing cells (S phase) in which to incorporate. Given the short half-life of azacytosines in the body, slow-growing tumors may require a longer dosing schedule, thereby increasing the possibility for treatment-related toxicity. One mode of circumventing this problem is through better drug delivery. For example, chemically modifying azanucleosides to improve their plasma stability and reduce drug degradation may allow the administration of lower doses over longer periods of time. To this end, decitabine can be contained within the dinucleotide S110, imparting it with a greater degree of resistance to the inactivating effects of deamination, while exhibiting comparable abilities for demethylation and inducing tumor cell growth inhibition as decitabine alone [129].

The inherent lack of specificity of azacitidine and decitabine for target genes allows for certain undesirable effects, e.g., global demethylation by azacitidine and decitabine may result in the expression of oncogenic loci and activation of transposable elements [130,131]. Decitabine has indeed been shown to induce the expression of *MDR1*, a gene implicated in drug resistance [132]. Furthermore, resistance to the cytotoxic effects of azacytosines has been noted regardless of degree or stability of incorporation into DNA, for various treatment regimens, and correlated with decreases in both drug-induced hypomethylation of long interspersed nuclear elements (LINEs) and levels of hENT1 [43,44,46,47,133,134]. Likewise, decreases in dCK and increases in cytidine deaminase may confer drug resistance [44,47,135,136].

6. Current Research Trends

The advent of epigenetic research of modified genes, regions of chromatin, histones, miRNA, and other modified/deregulated proteins in cancer has led to the discovery of many novel and useful

methylation biomarkers. Notably, decreased expression via hypermethylation of the $p15^{CDKN2B}$ cyclin-dependent kinase tumor suppressor gene is recognized in multiple cancers [137] including MDS, where its identification in early MDS is a marker of poor survival and transformation to AML [138]. P15 expression was increased in nine of 12 patients with hypermethylated *p15* upon treatment with decitabine, correlating with hematologic disease reversion including 3 complete responses [139]. Additionally, detection of methylated DNA in patient serum is minimally invasive and may be beneficial for novel biomarker identification and patient tumor characterization for tailoring therapy. Indeed, LINE-1 methylation levels as detected in plasma DNA may be a prognostic marker for chemosensitivity in patients with solid tumors [140].

MicroRNAs are a set of small (~22 nt) non-coding RNAs that bind the 3'-UTR of mRNAs and block translation. They are quickly becoming recognized as vital gene expression mediators during normal biological processes and disease states, including cancer. Blum *et al.* have reported on the response-predictive ability of *miR-29b* in older AML patients treated with decitabine [141]. A phase II clinical trial of single-agent decitabine in 53 older subjects (median 74 years; range, 60-85) with previously untreated CML resulted in an overall response rate of 64% (complete remission, 47%; incomplete remission, 17%) with overall survival for all subjects at 55 weeks (median disease-free survival those with CR, 46 weeks). Cycles of 20 mg/m^2 were variable and tailored to patient response and toxicities, responding patients typically having received one to two 10 day cycles (leading to incomplete CR) followed by one to two 4-5 day cycles (full CR). *MicroRNA-29b* (*miR-29b*) RNA levels as well as its mRNA targets, the DNA methyltransferases (*DNMT1*, *DNMT3A* and *DNMT3B*), were assayed for in 23 patients with available pre-treatment samples. *miR-29b* levels were significantly higher (P = 0.02) in responders (CR + incomplete CR; N = 14) than in non-responders (N = 9), while *DNMT3A*, the only target with differing expression, tended to be lower (P = 0.06) in responders than in non-responders. As DNMTs are important regulators of gene expression and protein function via gene promoter and protein methylation, respectively, in normal and cancer cells, microRNA thus represent an important upstream mediator of cancer development, progression and chemosensitivity.

Methylation frequency in a tumor sample may additionally be of diagnostic and prognostic value in multiple cancers. Indeed, Shen *et al.* have reported on the predictive value of a methylation profile of 10 genes in MDS patients, citing shorter median overall survival (12.3 *vs.* 17.5 months: P = 0.04) and progression-free survival (6.4 *vs.* 14.9 months; P = 0.009) in patients with greater methylation of these genes than in those with lower methylation (N = 89), further validated in two large patient cohorts (N = 228) [142]. Another recent publication relates the progression of MDS to AML to changing methylation patterns [143]. CpG methylation was more frequent and widespread than chromosomal aberrations in each of 184 MDS and AML patient bone marrow samples, meCpG frequency doubling from 6% of CpG loci in early, low-risk MDS to 12% after transformation to RAEB or AML. Furthermore, significantly more genes were methylated in RAEB/AML samples than in MDS or controls, including the independent prognostic marker frizzled-9 (*FZD9*), a Wnt/beta-catenin signaling receptor predictive of decreased survival in MDS/AML patients when methylated.

Novel uses for epigenetic drugs are also being identified. Radiosensitization using combinations of DNMT and HDAC inhibitors has been proposed based on the tumor-specific sensitization to therapeutics imparted by epigenetic drugs via induced tumor suppressor gene expression in epigenetically repressed chromatin, an uncommon occurrence in normal cells [144]. Administration of

oral decitabine at doses 17–34 times the optimal s.c. dose has been shown to reactivate fetal hemoglobin, demethylate the epsilon- and gamma-globin gene promoters, and increase histone acetylation of these promoters in baboons [145]. A recent publication by Garcia-Manero *et al.* reports on the feasibility for future cancer therapy of an oral azacitidine coated in a film to reduce its rapid breakdown in the body, thus increasing its bioavailability [146]. Celgene Corp. has recently included its own oral azacitidine in two phase I multicenter, open-label dose escalation trials for patients with MDS, CMML, AML, lymphoma, and multiple myeloma, assessing individual pharmacokinetics and pharmacodynamics alongside that of parenteral Vidaza® (www.clinicaltrials.gov, NCT00528983, NCT00761722).

7. Conclusions

The DNA methyltransferase inhibitors, azacitidine and decitabine, are two of a growing number of drugs designed to target epigenetic processes commonly deregulated during the development and progression of cancer. This class of compound has become a major contributor to basic and translational cancer research, easily one of the most valuable tools available for examining biological trends and implications of DNA methylation in normal and tumorigenic tissues. While they tend to exhibit a greater ability as solo agents to treat hematological malignancies than solid tumors, many groups are finding improvement in a multitude of cancers when combined with other agents, in particular with HDAC inhibitors, or when using different dosing schedules or modes of administration. As effective low doses allow for azacitidine and decitabine to be generally well tolerated, demethylating agents as a whole will continue to be utilized as and influence novel therapeutic interventions for cancer patients.

Acknowledgements

We received partial research funding for our clinical trial from Celgene Corportaion and Abraxis BioScience Inc.

References

1. Herman, J.G.; Baylin, S.B. Gene silencing in cancer in association with promoter hypermethylation. *New Engl. J. Med.* **2003**, *349*, 2042–2054.
2. Fleischhacker, M.; Schmidt, B. Circulating nucleic acids (CNAs) and cancer--a survey. *Biochim. Biophys. Acta* **2007**, *1775*, 181–232.
3. McCabe, M.T.; Brandes, J.C.; Vertino, P.M. Cancer DNA methylation: molecular mechanisms and clinical implications. *Clin. Cancer Res.* **2009**, *15*, 3927–3937.
4. Tost, J. Analysis of DNA methylation patterns for the early diagnosis, classification and therapy of human cancers. In *DNA Methylation Research Trends*; Kobayashi, T.B., Ed.; Nova Science Publishers: Hauppauge, NY, USA, 2007; pp. 87–133.
5. Tost, J. DNA methylation: an introduction to the biology and the disease-associated changes of a promising biomarker. *Mol. Biotechnol.* **2010**, *44*, 71–81.

6. Jones, P.L.; Veenstra, G.J.; Wade, P.A.; Vermaak, D.; Kass, S.U.; Landsberger, N.; Strouboulis, J.; Wolffe, A.P. Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Nat. Genet.* **1998**, *19*, 187–191.
7. D'Alessio, A.C.; Szyf, M. Epigenetic tête-à-tête: the bilateral relationship between chromatin modifications and DNA methylation. *Biochem. Cell Biol.* **2006**, *84*, 463–476.
8. Jair, K.W.; Bachman, K.E.; Suzuki, H.; Ting, A.H.; Rhee, I.; Yen, R.W.; Baylin, S.B.; Schuebel, K.E. De novo CpG island methylation in human cancer cells. *Cancer Res.* **2006**, *66*, 682–692.
9. Ting, A.H.; Jair, K.W.; Schuebel, K.E.; Baylin, S.B. Differential requirement for DNA methyltransferase 1 in maintaining human cancer cell gene promoter hypermethylation. *Cancer Res.* **2006**, *66*, 729–735.
10. Takai, D.; Jones, P.A. Comprehensive analysis of CpG islands in human chromosomes 21 and 22. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 3740–3745.
11. Wang, Y.; Leung, F.C. An evaluation of new criteria for CpG islands in the human genome as gene markers. *Bioinformatics* **2004**, *20*, 1170–1177.
12. Suzuki, M.M.; Bird, A. DNA methylation landscapes: provocative insights from epigenomics. *Nat. Rev. Genet.* **2008**, *9*, 465–476.
13. Geiman, T.M.; Muegge, K. DNA methylation in early development. *Mol. Reprod. Dev.* **2010**, *77*, 105–113.
14. Guibert, S.; Forne, T.; Weber, M. Dynamic regulation of DNA methylation during mammalian development. *Epigenomics* **2009**, *1*, 81–98.
15. Reik, W.; Dean, W.; Walter, J. Epigenetic reprogramming in mammalian development. *Science* **2001**, *293*, 1089–1093.
16. Goto, T.; Monk, M. Regulation of X-chromosome inactivation in development in mice and humans. *Microbiol. Mol. Biol. Rev.* **1998**, *62*, 362–378.
17. Barlow, D.P. Gametic imprinting in mammals. *Science (New York, N.Y.)* **1995**, *270*, 1610–1613.
18. Baylin, S.B. DNA methylation and gene silencing in cancer. *Nat. Clin. Pract. Oncol.* **2005**, *2* (Suppl. 1), S4–S11.
19. Esteller, M.; Garcia-Foncillas, J.; Andion, E.; Goodman, S.N.; Hidalgo, O.F.; Vanaclocha, V.; Baylin, S.B.; Herman, J.G. Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. *New Engl. J. Med.* **2000**, *343*, 1350–1354.
20. Hegi, M.E.; Diserens, A.C.; Gorlia, T.; Hamou, M.F.; de Tribolet, N.; Weller, M.; Kros, J.M.; Hainfellner, J.A.; Mason, W.; Mariani, L.; Bromberg, J.E.; Hau, P.; Mirimanoff, R.O.; Cairncross, J.G.; Janzer, R.C.; Stupp, R. MGMT gene silending and benefit from temozolomide in glioblastoma. *New Engl. J. Med.* **2005**, *352*, 997–1003.
21. Hegi, M.E.; Liu, L.; Herman, J.G., Stupp, R.; Wick, W.; Weller, M.; Mehta, M.P.; Gilbert, M.R. Correlation of O6-methylguanine methyltransferase (MGMT) promoter methylation with clinical outcomes in glioblastoma and clinical strategies to modulate MGMT activity. *J. Clin. Oncol.* **2008**, *26*, 4189–4199.
22. Weller, M.; Stupp, R.; Reifenberger, G.; Brandes, A.A.; van den Bent, M.J.; Wick, W.; Hegi, M.E. MGMT promoter methylation in malignant gliomas: ready for personalized medicine? *Nat. Rev. Neurol.* **2010**, *6*, 39–51.

23. Smith, J.F.; Mahmood, S.; Song, F.; Morrow, A.; Smiraglia, D.; Zhang, X.; Rajput, A.; Higgins, M.J.; Krumm, A.; Petrelli, N.J.; Costello, J.F.; Nagase, H.; Plass, C.; Held, W.A. Identification of DNA methylation in 3' genomic regions that are associated with upregulation of gene expression in colorectal cancer. *Epigenetics* **2007**, *2*, 161–172.
24. Weber, M.; Davies, J.J.; Wittig, D.; Oakeley, E.J.; Haase, M.; Lam, W.L.; Schubeler, D. Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells. *Nat. Genet.* **2005**, *37*, 853–862.
25. Mund, C.; Brueckner, B.; Lyko, F. Reactivation of epigenetically silenced genes by DNA methyltransferase inhibitors: basic concepts and clinical applications. *Epigenetics* **2006**, *1*, 7–13.
26. Feinberg, A.P.; Vogelstein, B. Hypomethylation distinguishes genes of some human cancers from their normal counterparts. *Nature* **1983**, *301*, 89–92.
27. Gama-Sosa, M.A.; Slagel, V.A.; Trewyn, R.W.; Oxenhandler, R.; Kuo, K.C.; Gehrke, C.W.; Ehrlich, M. The 5-methylcytosine content of DNA from human tumors. *Nucleic Acids Res.* **1983**, *11*, 6883–6894.
28. Lapeyre, J.N.; Becker, F.F. 5-Methylcytosine content of nuclear DNA during chemical hepatocarcinogenesis and in carcinomas which result. *Biochem. Biophys. Res. Commun.* **1979**, *87*, 698–705.
29. Lapeyre, J.N.; Walker, M.S.; Becker, F.F. DNA methylation and methylase levels in normal and malignant mouse hepatic tissues. *Carcinogenesis* **1981**, *2*, 873–878.
30. Zilberman, D.; Henikoff, S. Genome-wide analysis of DNA methylation patterns. *Development* **2007**, *134*, 3959–3965.
31. Costello, J.F.; Fruhwald, M.C.; Smiraglia, D.J.; Rush, L.J.; Robertson, G.P.; Gao, X.; Wright, F.A.; Feramisco, J.D.; Peltomaki, P.; Lang, J.C.; Schuller, D.E.; Yu, L.; Bloomfield, C.D.; Caligiuri, M.A.; Yates, A.; Nishikawa, R.; Su Huang, H.; Petrelli, N.J.; Zhang, X.; O'Dorisio, M.S.; Held, W.A.; Cavenee, W.K.; Plass, C. Aberrant CpG-island methylation has non-random and tumour-type-specific patterns. *Nat. Genet.* **2000**, *24*, 132–138.
32. Esteller, M. Cancer epigenomics: DNA methylomes and histone-modification maps. *Nat. Rev. Genet.* **2007**, *8*, 286–298.
33. Liu, S.; Ren, S.; Howell, P.; Fodstad, O.; Riker, A.I. Identification of novel epigenetically modified genes in human melanoma via promoter methylation gene profiling. *Pigment Cell Melanoma Res.* **2008**, *21*, 545–558.
34. Lujambio, A.; Calin, G.A.; Villanueva, A.; Ropero, S.; Sanchez-Cespedes, M.; Blanco, D.; Montuenga, L.M.; Rossi, S.; Nicoloso, M.S.; Faller, W.J.; Gallagher, W.M.; Eccles, S.A.; Croce, C.M.; Esteller, M. A microRNA DNA methylation signature for human cancer metastasis. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 13556–13561.
35. Lujambio, A.; Esteller, M. CpG island hypermethylation of tumor suppressor microRNAs in human cancer. *Cell Cycle* **2007**, *6*, 1455–1459.
36. McGarvey, K.M.; Van Neste, L.; Cope, L.; Ohm, J.E.; Herman, J.G.; Van Criekinge, W.; Schuebel, K.E.; Baylin, S.B. Defining a chromatin pattern that characterizes DNA-hypermethylated genes in colon cancer cells. *Cancer Res.* **2008**, *68*, 5753–5759.
37. Lehnertz, B.; Ueda, Y.; Derijck, A.A.; Braunschweig, U.; Perez-Burgos, L.; Kubicek, S.; Chen, T.; Li, E.; Jenuwein, T.; Peters, A.H. Suv39h-mediated histone H3 lysine 9 methylation directs

DNA methylation to major satellite repeats at pericentric heterochromatin. *Curr. Biol.* **2003**, *13*, 1192–1200.

38. Schlesinger, Y.; Straussman, R.; Keshet, I.; Farkash, S.; Hecht, M.; Zimmerman, J.; Eden, E.; Yakhini, Z.; Ben-Shushan, E.; Reubinoff, B.E.; Bergman, Y.; Simon, I.; Cedar, H. Polycomb-mediated methylation on Lys27 of histone H3 pre-marks genes for de novo methylation in cancer. *Nat. Genet.* **2007**, *39*, 232–236.
39. Filkowski, J.; Ilnytskyy, Y.; Tamminga, J.; Koturbash, I.; Golubov, A.; Bagnyukova, T.; Pogribny, I.; Kovalchuk, O. Hypomethylation and genome instability in the germline of exposed parents and their progeny is associated with altered miRNA expression. *Carcinogenesis* **2009**.
40. Khraiwesh, B.; Arif, M.A.; Seumel, G.I.; Ossowski, S.; Weigel, D.; Reski, R.; Frank, W. Transcriptional control of gene expression by microRNAs. *Cell* **2010**, *140*, 111–122.
41. Sinkkonen, L.; Hugenschmidt, T.; Berninger, P.; Gaidatzis, D.; Mohn, F.; Artus-Revel, C.G.; Zavolan, M.; Svoboda, P.; Filipowicz, W. MicroRNAs control de novo DNA methylation through regulation of transcriptional repressors in mouse embryonic stem cells. *Nat. Struct. Mol. Biol.* **2008**, *15*, 259–267.
42. Ting, A.H.; Suzuki, H.; Cope, L.; Schuebel, K.E.; Lee, B.H.; Toyota, M.; Imai, K.; Shinomura, Y.; Tokino, T.; Baylin, S.B. A requirement for DICER to maintain full promoter CpG island hypermethylation in human cancer cells. *Cancer Res.* **2008**, *68*, 2570–2575.
43. Huang, Y.; Anderle, P.; Bussey, K.J.; Barbacioru, C.; Shankavaram, U.; Dai, Z.; Reinhold, W.C.; Papp, A.; Weinstein, J.N.; Sadee, W. Membrane transporters and channels: role of the transportome in cancer chemosensitivity and chemoresistance. *Cancer Res.* **2004**, *64*, 4294–4301.
44. Qin, T.; Jelinek, J.; Si, J.; Shu, J.; Issa, J.P. Mechanisms of resistance to 5-aza-2'-deoxycytidine in human cancer cell lines. *Blood* **2009**, *113*, 659–667.
45. Rius, M.; Stresemann, C.; Keller, D.; Brom, M.; Schirrmacher, E.; Keppler, D.; Lyko, F. Human concentrative nucleoside transporter 1-mediated uptake of 5-azacytidine enhances DNA demethylation. *Mol. Cancer Ther.* **2009**, *8*, 225–231.
46. Christman, J.K. 5-Azacytidine and 5-aza-2'-deoxycytidine as inhibitors of DNA methylation: mechanistic studies and their implications for cancer therapy. *Oncogene* **2002**, *21*, 5483–5495.
47. Flatau, E.; Gonzales, F.A.; Michalowsky, L.A.; Jones, P.A. DNA methylation in 5-aza-2'-deoxycytidine-resistant variants of C3H 10T1/2 C18 cells. *Mol. Cell Biol.* **1984**, *4*, 2098–2102.
48. Momparler, R.L.; Bouchard, J.; Onetto, N.; Rivard, G.E. 5-aza-2'-deoxycytidine therapy in patients with acute leukemia inhibits DNA methylation. *Leukemia* **1984**, *8*, 181–185.
49. Chabner, B.A.; Drake, J.C.; Johns, D.G. Deamination of 5-azacytidine by a human leukemia cell cytidine deaminase. *Biochem. Pharmacol.* **1973**, *22*, 2763–2765.
50. van Groeningen, C.J.; Leyva, A.; O'Brien, A.M.; Gall, H.E.; Pinedo, H.M. Phase I and pharmacokinetic study of 5-aza-2'-deoxycytidine (NSC 127716) in cancer patients. *Cancer Res.* **1986**, *46*, 4831–4836.
51. Creusot, F.; Acs, G.; Christman, J.K. Inhibition of DNA methyltransferase and induction of Friend erythroleukemia cell differentiation by 5-azacytidine and 5-aza-2'-deoxycytidine. *J. Biol. Chem.* **1982**, *257*, 2041–2048.
52. Ghoshal, K.; Datta, J.; Majumder, S.; Bai, S.; Kutay, H.; Motiwala, T.; Jacob, S.T. 5-Aza-deoxycytidine induces selective degradation of DNA methyltransferase 1 by a proteasomal

pathway that requires the KEN box, bromo-adjacent homology domain, and nuclear localization signal. *Mol. Cell Biol.* **2005**, *25*, 4727–4741.

53. Stresemann, C.; Lyko, F. Modes of action of the DNA methyltransferase inhibitors azacytidine and decitabine. *Int. J. Cancer* **2008**, *123*, 8–13.
54. Liu, Z.; Liu, S.; Xie, Z.; Blum, W.; Perrotti, D.; Paschka, P.; Klisovic, R.; Byrd, J.; Chan, K.K.; Marcucci, G. Characterization of *in vitro* and *in vivo* hypomethylating effects of decitabine in acute myeloid leukemia by a rapid, specific and sensitive LC-MS/MS method. *Nuclecic Acids Res.* **2007**, *35*, e31.
55. Rountree, M.R.; Bachman, K.E.; Baylin, S.B. DNMT1 binds HDAC2 and a new co-repressor, DMAP1, to form a complex at replication foci. *Nat. Genet.* **2000**, *25*, 269–277.
56. Tatematsu, K.I.; Yamazaki, T.; Ishikawa, F. MBD2-MBD3 complex binds to hemi-methylated DNA and forms a complex containing DNMT1 at the replication foci in late S phase. *Genes Cells* **2000**, *5*, 677–688.
57. Juttermann, R.; Li, E.; Jaenisch, R. Toxicity of 5-aza-2'-deoxycytidine to mammalian cells is mediated primarily by covalent trapping of DNA methyltransferase rather than DNA demethylation. *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 11797–11801.
58. Issa, J.P.; Kantarjian, H.M. Targeting DNA methylation. *Clin. Cancer Res.* **2009**, *15*, 3938-3946.
59. Von Hoff, D.D.; Slavik, M.; Muggia, F.M. 5-Azacytidine. A new anticancer drug with effectiveness in acute myelogenous leukemia. *Ann. Intern. Med.* **1976**, *85*, 237–245.
60. Sorm, F.; Piskala, A.; Cihak, A.; Vesely, J. 5-Azacytidine, a new, highly effective cancerostatic. *Experientia* **1964**, *20*, 202–203.
60. Sorm, F.; Vesely, J. Effect of 5-aza-2'-deoxycytidine against leukemic and hemopoietic tissues in AKR mice. *Neoplasma* **1968**, *15*, 339–343.
62. Constantinides, P.G.; Jones, P.A.; Gevers, W. Funtional striated muscle cells from non-myoblast precursors following 5-azacytidine treatment. *Nature* **1977**, *267*, 364–366.
63. Taylor, S.M.; Jones, P.A. Multiple new phenotypes induced in 10T1/2 and 3T3 cells treated with 5-azacytidine. *Cell* **1979**, *17*, 771–779.
64. Jones, P.A. Effects of 5-azacytidine and its 2'-deoxyderivative on cell differentiation and DNA methylation. *Pharmacol. Ther.* **1985**, *28*, 17–27.
65. Jones, P.A.; Taylor, S.M. Cellular differentiation, cytidine analogs and DNA methylation. *Cell* **1980**, *20*, 85–93.
66. Issa, J.P.; Baylin, S.B.; Herman, J.G. DNA methylation changes in hematologic malignancies: biologic and clinical implications. *Leukemia* **1997**, *11*(Suppl. 1), S7–S11.
67. Ley, T.J.; DeSimone, J.; Anagnou, N.P.; Keller, G.H.; Humphries, R.K.; Turner, P.H.; Young, N.S.; Keller, P.; Nienhuis, A.W. 5-azacytidine selectively increases gamma-globin synthesis in a patient with beta+ thalassemia. *New Engl. J. Med.* **1982**, *307*, 1469–1475.
68. Charache, S.; Dover, G.; Smith, K.; Talbot, C.C., Jr.; Moyer, M.; Boyer, S. Treatment of sickle cell anemia with 5-azacytidine results in increased fetal hemoglobin production and is associated with nonrandom hypomethylation of DNA around the gamma-delta-beta-globin gene complex. *Proc. Natl. Acad. Sci. USA* **1983**, *80*, 4842–4846.

69. Pinto, A.; Attadia, V.; Fusco, A.; Ferrara, F.; Spada, O.A.; Di Fiore, P.P. 5-Aza-2'-deoxycytidine induces terminal differentiation of leukemic blasts from patients with acute myeloid leukemias. *Blood* **1984**, *64*, 922–929.
70. Momparler, R.L.; Gonzales, F.A. Effect of intravenous infusion of 5-aza-2'-deoxycytidine on survival time of mice with L1210 leukemia. *Cancer Res.* **1978**, *38*, 2673–2678.
71. Bellet, R.E.; Mastrangelo, M.J.; Engstrom, P.F.; Custer, R.P. Hepatotoxicity of 5-azacytidine (NSC-102816) (a clinical and pathologic study). *Neoplasma* **1973**, *20*, 303–309.
72. Santini, V.; Kantarjian, H.M.; Issa, J.P. Changes in DNA methylation in neoplasia: pathophysiology and therapeutic implications. *Ann. Intern. Med.* **2001**, *134*, 573–586.
73. Tefferi, A.; Vardiman, J.W. Myelodysplastic syndromes. *New Engl. J. Med.* **2009**, *361*, 1872–1885.
74. Bennett, J.M.; Catovsky, D.; Daniel, M.T.; Flandrin, G.; Galton, D.A.; Gralnick, H.R.; Sultan, C. Proposals for the classification of the myelodysplastic syndromes. *Br. J. Haematol.* **1982**, *51*, 189–199.
75. Bennett, J.M.; Kouides, P.A.; Forman, S.J. The myelodysplastic syndromes: morphology, risk assessment, and clinical management (2002). *Int. J. Hematol.* **2002**, *76* (Suppl. 2), 228–238.
76. Cheson, B.D.; Bennett, J.M.; Kantarjian, H.; Pinto, A.; Schiffer, C.A.; Nimer, S.D.; Lowenberg, B.; Beran, M.; de Witte, T.M.; Stone, R.M.; Mittelman, M.; Sanz, G.F.; Wijermans, P.W.; Gore, S.; Greenberg, P.L. Report of an international working group to standardize response criteria for myelodysplastic syndromes. *Blood* **2000**, *96*, 3671–3674.
77. Germing, U.; Gattermann, N.; Strupp, C.; Aivado, M.; Aul, C. Validation of the WHO proposals for a new classification of primary myelodysplastic syndromes: a retrospective analysis of 1600 patients. *Leukemia* **2000**, *24*, 983–992.
78. Harris, N.L.; Jaffe, E.S.; Diebold, J.; Flandrin, G.; Muller-Hermelink, H.K.; Vardiman, J.; Lister, T.A.; Bloomfield, C.D. World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues: report of the Clinical Advisory Committee meeting-Airlie House, Virginia, November 1997. *J. Clin. Oncol.* **1999**, *17*, 3835–3849.
79. Vardiman, J.W.; Thiele, J.; Arber, D.A.; Brunning, R.D.; Borowitz, M.J.; Porwit, A.; Harris, N.L.; Le Beau, M.M.; Hellstrom-Lindberg, E.; Tefferi, A.; Bloomfield, C.D. The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. *Blood* **2009**, *114*, 937–951.
80. Mills, K.I.; Kohlmann, A.; Williams, P.M.; Wieczorek, L.; Liu, W.M.; Li, R.; Wei, W.; Bowen, D.T.; Loeffler, H.; Hernandez, J.M.; Hofmann, W.K.; Haferlach, T. Microarray-based classifiers and prognosis models identify subgroups with distinct clinical outcomes and high risk of AML transformation of myelodysplastic syndrome. *Blood* **2009**, *114*, 1063–1072.
81. Silverman, L.R. Myelodysplastic syndrome. In *Cancer Medicine*; 5th ed.; Bast, R. J.; Kufe, D. W.; Pollock, R. E.; Weichselbaum, R. R.; Holland, J. F.; Frei, E.; Gansler, T. S., Eds.; B.C. Decker Inc.: Hamilton, Ontario, 2000; pp. 1931–1946.
82. Rollison, D.E.; Howlader, N.; Smith, M.T.; Strom, S.S.; Merritt, W.D.; Ries, L.A.; Edwards, B.K.; List, A.F. Epidemiology of myelodysplastic syndromes and chronic myeloproliferative disorders in the United States, 2001–2004, using data from the NAACCR and SEER programs. *Blood* **2008**, *112*, 45–52.

83. Warlick, E.D.; Cioc, A.; Defor, T.; Dolan, M.; Weisdorf, D. Allogeneic stem cell transplantation for adults with myelodysplastic syndromes: importance of pretransplant disease burden. *Biol. Blood Marrow Transplant.* **2009**, *15*, 30–38.
84. Cutler, C.S.; Lee, S.J.; Greenberg, P.; Deeg, H.J.; Perez, W.S.; Anasetti, C.; Bolwell, B.J.; Cairo, M.S.; Gale, R.P.; Klein, J.P.; Lazarus, H.M.; Liesveld, J.L.; McCarthy, P.L.; Milone, G.A.; Rizzo, J.D.; Schultz, K.R.; Trigg, M.E.; Keating, A.; Weisdorf, D.J.; Antin, J.H.; Horowitz, M.M. A decision analysis of allogeneic bone marrow transplantation for the myelodysplastic syndromes: delayed transplantation for low-risk myelodysplasia is associated with improved outcome. *Blood* **2004**, *104*, 579–585.
85. Silverman, L.R.; Demakos, E.P.; Peterson, B.L.; Kornblith, A.B.; Holland, J.C.; Odchimar-Reissig, R.; Stone, R.M.; Nelson, D.; Powell, B.L.; DeCastro, C.M.; Ellerton, J.; Larson, R.A.; Schiffer, C.A.; Holland, J.F. Randomized controlled trial of azacitidine in patients with the myelodysplastic syndrome: a study of the cancer and leukemia group B. *J. Clin. Oncol.* **2002**, *20*, 2429–2440.
86. De Padua Silva, L.; de Lima, M.; Kantarjian, H.; Faderl, S.; Kebriaei, P.; Giralt, S.; Davisson, J.; Garcia-Manero, G.; Champlin, R.; Issa, J.P.; Ravandi, F. Feasibility of allo-SCT after hypomethylating therapy with decitabine for myelodysplastic syndrome. *Bone Marrow Transplant.* **2009**, *43*, 839–843.
87. Lubbert, M.; Bertz, H.; Ruter, B.; Marks, R.; Claus, R.; Wasch, R.; Finke, J. Non-intensive treatment with low-dose 5-aza-2'-deoxycytidine (DAC) prior to allogeneic blood SCT of older MDS/AML patients. *Bone Marrow Transplant.* **2009**, *44*, 585–588.
88. Silverman, L.; Holland, J.F.; Demakos, E.P. Azacitidine in myelodysplastic syndromes: CALGB studies 8421 and 8921. *Ann. Hematol.* **1994**, *68*, A12.
89. Silverman, L.R.; Holland, J.F.; Weinberg, R.S.; Alter, B.P.; Davis, R.B.; Ellison, R.R.; Demakos, E.P.; Cornell, C.J., Jr.; Carey, R.W.; Schiffer, C.; *et al.* Effects of treatment with 5-azacytidine on the *in vivo* and *in vitro* hematopoiesis in patients with myelodysplastic syndromes. *Leukemia* **1993**, *7* (Suppl. 1), 21–29.
90. Silverman, L.R.; McKenzie, D.R.; Peterson, B.L.; Holland, J.F.; Backstrom, J.T.; Beach, C.L.; Larson, R.A. Further analysis of trials with azacitidine in patients with myelodysplastic syndrome: studies 8421, 8921, and 9221 by the Cancer and Leukemia Group B. *J. Clin. Oncol.* **2006**, *24*, 3895–3903.
91. Marcucci, G.; Silverman, L.; Eller, M.; Lintz, L.; Beach, C.L. Bioavailability of azacitidine subcutaneous *versus* intravenous in patients with the myelodysplastic syndromes. *J. Clin. Pharmacol.* **2005**, *45*, 597–602.
92. Kaminskas, E.; Farrell, A.; Abraham, S.; Baird, A.; Hsieh, L.S.; Lee, S.L.; Leighton, J.K.; Patel, H.; Rahman, A.; Sridhara, R.; Wang, Y.C.; Pazdur, R. Approval summary: azacitidine for treatment of myelodysplastic syndrome subtypes. *Clin. Cancer Res.* **2005**, *11*, 3604–3608.
93. Kornblith, A.B.; Herndon, J.E., 2nd; Silverman, L.R.; Demakos, E.P.; Odchimar-Reissig, R.; Holland, J.F.; Powell, B.L.; DeCastro, C.; Ellerton, J.; Larson, R.A.; Schiffer, C.A.; Holland, J.C. Impact of azacytidine on the quality of life of patients with myelodysplastic syndrome treated in a randomized phase III trial: a Cancer and Leukemia Group B study. *J. Clin. Oncol.* **2002**, *20*, 2441–2452.

94. Momparler, R.L.; Rivard, G.E.; Gyger, M. Clinical trial on 5-aza-2'-deoxycytidine in patients with acute leukemia. *Pharmacol. Ther.* **1985**, *30*, 277–286.
95. Wijermans, P.; Lubbert, M.; Verhoef, G.; Bosly, A.; Ravoet, C.; Andre, M.; Ferrant, A. Low-dose 5-aza-2'-deoxycytidine, a DNA hypomethylating agent, for the treatment of high-risk myelodysplastic syndrome: a multicenter phase II study in elderly patients. *J. Clin. Oncol.* **2000**, *18*, 956–962.
96. Wijermans, P.W.; Krulder, J.W.; Huijgens, P.C.; Neve, P. Continuous infusion of low-dose 5-Aza-2'-deoxycytidine in elderly patients with high-risk myelodysplastic syndrome. *Leukemia* **1997**, *11*, 1–5.
97. Kantarjian, H.; Issa, J.P.; Rosenfeld, C.S.; Bennett, J.M.; Albitar, M.; DiPersio, J.; Klimek, V.; Slack, J.; de Castro, C.; Ravandi, F.; Helmer, R., 3rd; Shen, L.; Nimer, S.D.; Leavitt, R.; Raza, A.; Saba, H. Decitabine improves patient outcomes in myelodysplastic syndromes: results of a phase III randomized study. *Cancer* **2006**, *106*, 1794–1803.
98. Gore, S.D.; Jones, C.; Kirkpatrick, P. Decitabine. *Nat. Rev. Drug Discov.* **2006**, *5*, 891–892.
99. Steensma, D.P.; Baer, M.R.; Slack, J.L.; Buckstein, R.; Godley, L.A.; Garcia-Manero, G.; Albitar, M.; Larsen, J.S.; Arora, S.; Cullen, M.T.; Kantarjian, H. Multicenter study of decitabine administered daily for 5 days every 4 weeks to adults with myelodysplastic syndromes: the alternative dosing for outpatient treatment (ADOPT) trial. *J. Clin. Oncol.* **2009**, *27*, 3842–3848.
100. Fenaux, P.; Mufti, G.J.; Hellstrom-Lindberg, E.; Santini, V.; Finelli, C.; Giagounidis, A.; Schoch, R.; Gattermann, N.; Sanz, G.; List, A.; Gore, S.D.; Seymour, J.F.; Bennett, J.M.; Byrd, J.; Backstrom, J.; Zimmerman, L.; McKenzie, D.; Beach, C.; Silverman, L.R. Efficacy of azacitidine compared with that of conventional care regimens in the treatment of higher-risk myelodysplastic syndromes: a randomised, open-label, phase III study. *Lancet Oncol.* **2009**, *10*, 223–232.
101. Griffiths, E.A.; Gore, S.D. DNA methyltransferase and histone deacetylase inhibitors in the treatment of myelodysplastic syndromes. *Semin. Hematol.* **2008**, *45*, 23–30.
102. Gurion, R.; Vidal, L.; Gafter-Gvili, A.; Belnik, Y.; Yeshurun, M.; Raanani, P.; Shpilberg, O. 5-azacitidine prolongs overall survival in patients with myelodysplastic syndrome--a systematic review and meta-analysis. *Haematologica* **2010**, *95*, 303–310.
103. Sharma, S.; Kelly, T.K.; Jones, P.A. Epigenetics in cancer. *Carcinogenesis* **2010**, *31*, 27–36.
104. Abele, R.; Clavel, M.; Dodion, P.; Bruntsch, U.; Gundersen, S.; Smyth, J.; Renard, J.; van Glabbeke, M.; Pinedo, H.M. The EORTC Early Clinical Trials Cooperative Group experience with 5-aza-2'-deoxycytidine (NSC 127716) in patients with colo-rectal, head and neck, renal carcinomas and malignant melanomas. *Eur. J. Cancer Clin. Oncol.* **1987**, *23*, 1921–1924.
105. Appleton, K.; Mackay, H.J.; Judson, I.; Plumb, J.A.; McCormick, C.; Strathdee, G.; Lee, C.; Barrett, S.; Reade, S.; Jadayel, D.; Tang, A.; Bellenger, K.; Mackay, L.; Setanoians, A.; Schatzlein, A.; Twelves, C.; Kaye, S.B.; Brown, R. Phase I and pharmacodynamic trial of the DNA methyltransferase inhibitor decitabine and carboplatin in solid tumors. *J. Clin. Oncol.* **2007**, *25*, 4603–4609.
106. Bellet, R.E.; Mastrangelo, M.J.; Engstrom, P.F.; Strawitz, J.G.; Weiss, A.J.; Yarbro, J.W. Clinical trial with subcutaneously administered 5-azacytidine (NSC-102816). *Cancer Chemother. Rep.* **1974**, *58*, 217–222.

107. Cunningham, T.J.; Nemoto, T.; Rosner, D.; Knight, E.; Taylor, S.; Rosenbaum, C.; Horton, J.; Dao, T. Comparison of 5-azacytidine (NSC-102816) with CCNU (NSC-79037) in the treatment of patients with breast cancer and evaluation of the subsequent use of cyclophosphamide (NSC-26271). *Cancer Chemother. Rep.* **1974**, *58*, 677–681.
108. Moertel, C.G.; Schutt, A.J.; Reitemeier, R.J.; Hahn, R.G. Phase II study of 5-azacytidine (NSC-102816) in the treatment of advanced gastrointestinal cancer. *Cancer Chemother. Rep.* **1972**, *56*, 649–652.
109. Momparler, R.L.; Bouffard, D.Y.; Momparler, L.F.; Dionne, J.; Belanger, K.; Ayoub, J. Pilot phase I-II study on 5-aza-2'-deoxycytidine (Decitabine) in patients with metastatic lung cancer. *Anticancer Drugs* **1997**, *8*, 358–368.
110. Samuels, B.L.; Herndon, J.E., 2nd; Harmon, D.C.; Carey, R.; Aisner, J.; Corson, J.M.; Suzuki, Y.; Green, M.R.; Vogelzang, N.J. Dihydro-5-azacytidine and cisplatin in the treatment of malignant mesothelioma: a phase II study by the Cancer and Leukemia Group B. *Cancer* **1998**, *82*, 1578–1584.
111. Sessa, C.; ten Bokkel Huinink, W.; Stoter, G.; Renard, J.; Cavalli, F. Phase II study of 5-aza-2'-deoxycytidine in advanced ovarian carcinoma. The EORTC Early Clinical Trials Group. *Eur. J. Cancer* **1990**, *26*, 137–138.
112. Thibault, A.; Figg, W.D.; Bergan, R.C.; Lush, R.M.; Myers, C.E.; Tompkins, A.; Reed, E.; Samid, D. A phase II study of 5-aza-2'deoxycytidine (decitabine) in hormone independent metastatic (D2) prostate cancer. *Tumori* **1998**, *84*, 87–89.
113. Weiss, A.J.; Metter, G.E.; Nealon, T.F.; Keanan, J.P.; Ramirez, G.; Swaiminathan, A.; Fletcher, W.S.; Moss, S.E.; Manthei, R.W. Phase II study of 5-azacytidine in solid tumors. *Cancer Treat. Rep.* **1977**, *61*, 55–58.
114. Weiss, A.J.; Stambaugh, J.E.; Mastrangelo, M.J.; Laucius, J.F.; Bellet, R.E. Phase I study of 5-azacytidine (NSC-102816). *Cancer Chemother. Rep.* **1972**, *56*, 413–419.
115. Aparicio, A.; Weber, J.S. Review of the clinical experience with 5-azacytidine and 5-aza-2'-deoxycytidine in solid tumors. *Curr. Opin. Investig. Drugs* **2002**, *3*, 627–633.
116. Hellebrekers, D.M.; Jair, K.W.; Vire, E.; Eguchi, S.; Hoebers, N.T.; Fraga, M.F.; Esteller, M.; Fuks, F.; Baylin, S.B.; van Engeland, M.; Griffioen, A.W. Angiostatic activity of DNA methyltransferase inhibitors. *Mol. Cancer Ther.* **2006**, *5*, 467–475.
117. Samlowski, W.E.; Leachman, S.A.; Wade, M.; Cassidy, P.; Porter-Gill, P.; Busby, L.; Wheeler, R.; Boucher, K.; Fitzpatrick, F.; Jones, D.A.; Karpf, A.R. Evaluation of a 7-day continuous intravenous infusion of decitabine: inhibition of promoter-specific and global genomic DNA methylation. *J. Clin. Oncol.* **2005**, *23*, 3897–3905.
118. Aparicio, A.; Eads, C.A.; Leong, L.A.; Laird, P.W.; Newman, E.M.; Synold, T.W.; Baker, S.D.; Zhao, M.; Weber, J.S. Phase I trial of continuous infusion 5-aza-2'-deoxycytidine. *Cancer Chemother. Pharmacol.* **2003**, *51*, 231–239.
119. Beyrouthy, M.J.; Garner, K.M.; Hever, M.P.; Freemantle, S.J.; Eastman, A.; Dmitrovsky, E.; Spinella, M.J. High DNA methyltransferase 3B expression mediates 5-aza-deoxycytidine hypersensitivity in testicular germ cell tumors. *Cancer Res.* **2009**, *69*, 9360–9366.
120. Sonpavde, G.; Aparicio, A.M.; Zhan, F.; North, B.; Delaune, R.; Garbo, L.E.; Rousey, S.R.; Weinstein, R.E.; Xiao, L.; Boehm, K.A.; Asmar, L.; Fleming, M.T.; Galsky, M.D.; Berry, W.R.;

Von Hoff, D.D. Azacitidine favorably modulates PSA kinetics correlating with plasma DNA LINE-1 hypomethylation in men with chemonaive castration-resistant prostate cancer. *Urol. Oncol.* **2009**, doi:10.1016/j.urolonc.2009.09.015.

121. Gradishar, W.J. Albumin-bound paclitaxel: a next-generation taxane. *Expert Opin. Pharmacother.* **2006**, *7*, 1041–1053.
122. Ito, M.; Ito, G.; Kondo, M.; Uchiyama, M.; Fukui, T.; Mori, S.; Yoshioka, H.; Ueda, Y.; Shimokata, K.; Sekido, Y. Frequent inactivation of RASSF1A, BLU, and SEMA3B on 3p21.3 by promoter hypermethylation and allele loss in non-small cell lung cancer. *Cancer Lett.* **2005**, *225*, 131–139.
123. Mok, S.C.; Chan, W.Y.; Wong, K.K.; Muto, M.G.; Berkowitz, R.S. SPARC, an extracellular matrix protein with tumor-suppressing activity in human ovarian epithelial cells. *Oncogene* **1996**, *12*, 1895–1901.
124. Sato, N.; Fukushima, N.; Maehara, N.; Matsubayashi, H.; Koopmann, J.; Su, G.H.; Hruban, R.H.; Goggins, M. SPARC/osteonectin is a frequent target for aberrant methylation in pancreatic adenocarcinoma and a mediator of tumor-stromal interactions. *Oncogene* **2003**, *22*, 5021–5030.
125. Sova, P.; Feng, Q.; Geiss, G.; Wood, T.; Strauss, R.; Rudolf, V.; Lieber, A.; Kiviat, N. Discovery of novel methylation biomarkers in cervical carcinoma by global demethylation and microarray analysis. *Cancer Epidemiol. Biomarkers Prev.* **2006**, *15*, 114–123.
126. Suzuki, M.; Hao, C.; Takahashi, T.; Shigematsu, H.; Shivapurkar, N.; Sathyanarayana, U.G.; Iizasa, T.; Fujisawa, T.; Hiroshima, K.; Gazdar, A.F. Aberrant methylation of SPARC in human lung cancers. *Br. J. Cancer* **2005**, *92*, 942–948.
127. Yang, E.; Kang, H.J.; Koh, K.H.; Rhee, H.; Kim, N.K.; Kim, H. Frequent inactivation of SPARC by promoter hypermethylation in colon cancers. *Int. J. Cancer* **2007**, *121*, 567–575.
128. Koblinski, J.E.; Kaplan-Singer, B.R.; VanOsdol, S.J.; Wu, M.; Engbring, J.A.; Wang, S.; Goldsmith, C.M.; Piper, J.T.; Vostal, J.G.; Harms, J.F.; Welch, D.R.; Kleinman, H.K. Endogenous osteonectin/SPARC/BM-40 expression inhibits MDA-MB-231 breast cancer cell metastasis. *Cancer Res.* **2005**, *65*, 7370–7377.
129. Yoo, C.B.; Jeong, S.; Egger, G.; Liang, G.; Phiasivongsa, P.; Tang, C.; Redkar, S.; Jones, P.A. Delivery of 5-aza-2'-deoxycytidine to cells using oligodeoxynucleotides. *Cancer Res.* **2007**, *67*, 6400–6408.
130. Gaudet, F.; Hodgson, J.G.; Eden, A.; Jackson-Grusby, L.; Dausman, J.; Gray, J.W.; Leonhardt, H.; Jaenisch, R. Induction of tumors in mice by genomic hypomethylation. *Science* **2003**, *300*, 489–492.
131. Howard, G.; Eiges, R.; Gaudet, F.; Jaenisch, R.; Eden, A. Activation and transposition of endogenous retroviral elements in hypomethylation induced tumors in mice. *Oncogene* **2008**, *27*, 404–408.
132. Kantharidis, P.; El-Osta, A.; deSilva, M.; Wall, D.M.; Hu, X.F.; Slater, A.; Nadalin, G.; Parkin, J.D.; Zalcberg, J.R. Altered methylation of the human MDR1 promoter is associated with acquired multidrug resistance. *Clin. Cancer Res.* **1997**, *3*, 2025–2032.
133. Hubeek, I.; Stam, R.W.; Peters, G.J.; Broekhuizen, R.; Meijerink, J.P.; van Wering, E.R.; Gibson, B.E.; Creutzig, U.; Zwaan, C.M.; Cloos, J.; Kuik, D.J.; Pieters, R.; Kaspers, G.J. The human equilibrative nucleoside transporter 1 mediates *in vitro* cytarabine sensitivity in childhood acute myeloid leukaemia. *Br. J. Cancer* **2005**, *93*, 1388–1394.

134. Lemaire, M.; Chabot, G.G.; Raynal, N.J.; Momparler, L.F.; Hurtubise, A.; Bernstein, M.L.; Momparler, R.L. Importance of dose-schedule of 5-aza-2'-deoxycytidine for epigenetic therapy of cancer. *BMC Cancer* **2008**, *8*, 128.
135. Adams, R.L.; Fulton, J.; Kirk, D. The effect of 5-azadeoxycytidine on cell growth and DNA methylation. *Biochim. Biophys. Acta.* **1982**, *697*, 286–294.
136. Stegmann, A.P.; Honders, M.W.; Willemze, R.; Landegent, J.E. De novo induced mutations in the deoxycytidine kinase (dck) gene in rat leukemic clonal cell lines confer resistance to cytarabine (AraC) and 5-aza-2'-deoxycytidine (DAC). *Leukemia* **1995**, *9*, 1032–1038.
137. Park, M.T.; Lee, S.J. Cell cycle and cancer. *J. Biochem. Mol. Biol.* **2003**, *36*, 60–65.
138. Quesnel, B.; Guillerm, G.; Vereecque, R.; Wattel, E.; Preudhomme, C.; Bauters, F.; Vanrumbeke, M.; Fenaux, P. Methylation of the p15(INK4b) gene in myelodysplastic syndromes is frequent and acquired during disease progression. *Blood* **1998**, *91*, 2985–2990.
139. Daskalakis, M.; Nguyen, T.T.; Nguyen, C.; Guldberg, P.; Kohler, G.; Wijermans, P.; Jones, P.A.; Lubbert, M. Demethylation of a hypermethylated P15/INK4B gene in patients with myelodysplastic syndrome by 5-Aza-2'-deoxycytidine (decitabine) treatment. *Blood* **2002**, *100*, 2957–2964.
140. Aparicio, A.; North, B.; Barske, L.; Wang, X.; Bollati, V.; Weisenberger, D.; Yoo, C.; Tannir, N.; Horne, E.; Groshen, S.; Jones, P.; Yang, A.; Issa, J.P. LINE-1 methylation in plasma DNA as a biomarker of activity of DNA methylation inhibitors in patients with solid tumors. *Epigenetics* **2009**, *4*, 176–184.
141. Blum, W.; Garzon, R.; Klisovic, R.B.; Schwind, S.; Walker, A.; Geyer, S.; Liu, S.; Havelange, V.; Becker, H.; Schaaf, L.; Mickle, J.; Devine, H.; Kefauver, C.; Devine, S.M.; Chan, K.K.; Heerema, N.A.; Bloomfield, C.D.; Grever, M.R.; Byrd, J.C.; Villalona-Calero, M.; Croce, C.M.; Marcucci, G. Clinical response and miR-29b predictive significance in older AML patients treated with a 10-day schedule of decitabine. *Proc. Natl. Acad. Sci. U S A* **2010**, *107*, 7473–7478.
142. Shen, L.; Kantarjian, H.; Guo, Y.; Lin, E.; Shan, J.; Huang, X.; Berry, D.; Ahmed, S.; Zhu, W.; Pierce, S.; Kondo, Y.; Oki, Y.; Jelinek, J.; Saba, H.; Estey, E.; Issa, J.P. DNA methylation predicts survival and response to therapy in patients with myelodysplastic syndromes. *J. Clin. Oncol.* **2010**, *28*, 605–613.
143. Jiang, Y.; Dunbar, A.; Gondek, L.P.; Mohan, S.; Rataul, M.; O'Keefe, C.; Sekeres, M.; Saunthararajah, Y.; Maciejewski, J.P. Aberrant DNA methylation is a dominant mechanism in MDS progression to AML. *Blood* **2009**, *113*, 1315–1325.
144. De Schutter, H.; Nuyts, S. Radiosensitizing potential of epigenetic anticancer drugs. *Anticancer Agents Med. Chem.* **2009**, *9*, 99–108.
145. Lavelle, D.; Chin, J.; Vaitkus, K.; Redkar, S.; Phiasivongsa, P.; Tang, C.; Will, R.; Hankewych, M.; Roxas, B.; Singh, M.; Saunthararajah, Y.; Desimone, J. Oral decitabine reactivates expression of the methylated gamma-globin gene in Papio anubis. *Am. J. Hematol.* **2007**, *82*, 981–985.
146. Garcia-Manero, G.; Stoltz, M.L.; Ward, M.R.; Kantarjian, H.; Sharma, S. A pilot pharmacokinetic study of oral azacitidine. *Leukemia* **2008**, *22*, 1680–1684.

XP-002581313

JOURNAL OF CLINICAL ONCOLOGY
Official Journal of the American Society of Clinical Oncology

Journal of Clinical Oncology, 2009 ASCO Annual Meeting Proceedings (Post-Meeting Edition).
Vol 27, No 15S (May 20 Supplement), 2009: 8055

Abstract

Interim analysis of a phase II trial of 5-azacitidine (5AC) and entinostat (SNDX-275) in relapsed advanced lung cancer (NSCLC)

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8055

Background: Epigenetic gene silencing mediated through aberrant DNA methylation and histone deacetylation is a key contributor to lung carcinogenesis. Pre-clinical studies suggest that combining inhibitors of DNA methyltransferase (DNMT) with inhibitors of histone deacetylase (HDAC) synergistically induce re-expression of epigenetically-silenced tumor suppressor genes. Clinical studies at our institution combining the DNMT inhibitor, 5AC, with the HDAC inhibitor, entinostat, in hematologic malignancies have shown remarkable clinical activity. We hypothesized a similar effect would be seen in lung cancer. This study aims to assess the response rate and time to progression of 5AC and entinostat in NSCLC. **Methods:** Patients (pts) include adults with histologically confirmed recurrent NSCLC and progressive disease after ≥1 prior chemotherapy regimen. 40 mg/m^2 of 5AC is administered SQ days 1-6 and 8-10 with 7 mg of entinostat PO days 3 and 10 of a 28 day cycle. A standard Simon two stage design is being used. The sample size is calculated with a power of 90% and a two-sided type 1 error allowance of 5%. Stage 1 included 18 pts, with subsequent expansion to a total of 32. **Results:** 25 pts have enrolled to date. Demographic characteristics include: mean age (range) - 63 (46- 80); M:F - 1:2; 80% former smokers; 80% adenocarcinomas; mean # of previous therapies - 3. One pt. has had a complete response. She remained on therapy for 14 m and came off therapy due to a new nodule which was resected. Molecular analysis suggests a second primary stage I NSCLC. She remains disease free at 20 m. Another man has stabilization of disease (SD) for ≥16 m with marked symptomatic improvement. Another pt had SD for 4 months. The remaining pts have progressed after 2 cycles of therapy. Main toxicities included injection site reactions, nausea/vomiting, constipation, fatigue, and hematologic toxicities. **Conclusions:** The combination of 5AC and entinostat is safe and well tolerated in advanced NSCLC pts. 2 pts have had durable benefit from treatment, including a complete response. Pharmacodynamic and pharmacokinetic analyses are being conducted to identify characteristics of the subset of pts responding to this novel therapy.

Author Disclosure

Employment or Leadership	Consultant or Advisory Role	Stock Ownership	Honoraria	Research Funding	Expert Testimony	Other Remuneration
		Oncomethylome Sciences	BioNumerik Pharmaceuticals, Constellation Pharmaceuticals,	BioNumerik Pharmaceuticals, Celgene, Oncomethylome		

Progen Sciences, Pharmion

Abstract presentation from the 2009 ASCO Annual Meeting

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Seminars in **Oncology**

Epigenetic Therapy of Cancer With 5-Aza-2′-Deoxycytidine (decitabine)

Richard L. Momparler

Epigenetic events, such as aberrant DNA methylation, have been demonstrated to silence the expression of many genes that suppress malignancy. Since the event is reversible, it is an interesting target for intervention with specific inhibitors of DNA methylation, such as 5-aza-2′-deoxycytidine (5-AZA-CdR, decitabine). 5-AZA-CdR is a prodrug that requires activation via phosphorylation by deoxycytidine kinase. The nucleotide analog is incorporated into DNA, where it produces an irreversible inactivation of DNA methyltransferase. 5-AZA-CdR is an S-phase–specific agent. The demethylation of DNA by this analog in neoplastic cells can lead to the reactivation of silent tumor-suppressor genes, induction of differentiation or senescence, growth inhibition, and loss of clonogenicity. 5-AZA-CdR was demonstrated to be a potent antineoplastic agent against leukemia and tumors in animal models. Preliminary clinical trials of 5-AZA-CdR using different dose-schedules have shown interesting antineoplastic activity in patients with leukemia, myelodysplastic syndrome (MDS), and non-small cell lung cancer (NSCLC). Pharmacokinetic studies have shown that 5-AZA-CdR has a short in vivo half-life of 15 to 25 minutes. The major toxicity produced by this analog is granulocytopenia. To exploit the full chemotherapeutic potential of 5-AZA-CdR for the treatment of cancer, its optimal dose-schedule has to be found. This will require a good understanding of the pharmacology of this analog and its action on both normal and neoplastic cells.
Semin Oncol 32:443-451

Aberrant DNA methylation is an epigenetic mechanism that can inactivate the expression of genes that suppress tumorigenesis.[1] Genes that are involved in every step of tumorigenesis can be silenced by this mechanism.[2] This epigenetic event that effects gene expression does not involve any change in DNA sequence. The genes involved include tumor-suppressor genes; genes that suppress apoptosis, metastasis, and angiogenesis; genes that repair DNA; and genes that express tumor-associated antigens (Table 1). The molecular mechanism of silencing gene expression appears to be due to the attachment of 5-methylcytosine binding proteins to the methylated promoter, which blocks the action of transcription factors.[14] Since this epigenetic change is reversible, it provides an interesting target for chemotherapeutic intervention. 5-aza-2′-deoxycytidine (decitabine, 5-AZA-CdR), a potent and specific inhibitor of DNA methylation, has been shown to reactivate the expression of genes that suppress malignancy[15] (Table 1). This observation, plus the reports that this nucleoside analog is a very potent antineoplastic agent against leukemia and tumors in animal models,[16,17] make it a very interesting agent to investigate for cancer therapy. In preliminary clinical trials in patients with hematologic malignancies and solid tumors, 5-AZA-CdR has shown promising antineoplastic activity.[18–20] However, the optimal dose-schedule of administration remains to be determined. Knowledge of the pharmacology of 5-AZA-CdR and its action on both normal malignant cells can be used to attain this objective.[21]

Pharmacology of 5-AZA-CdR

Metabolism and Molecular Mechanism of Action

5-AZA-CdR is a deoxycytidine analog in which the carbon at the 5 position of the pyrimidine ring is replaced by a nitrogen (Fig 1). This analog is a prodrug that is activated via phosphorylation by deoxycytidine kinase (Fig 2). Other kinases in the cell rapidly convert the monophosphate (5AZA-dCMP) to its triphosphate form (5AZA-dCTP), the active form of the

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0093-7754/05/$-see front matter
doi:10.1053/j.seminoncol.2005.07.008

Table 1 Reactivation of Different Classes of Genes That Suppress Malignancy by 5-AZA-CdR

Gene Function	Example*	References
Inhibit cell cycle progression	*p15CDKN2B; p16CDKN2A*	3,4
Inhibit tumor metastasis	*E-cadherin*	5
Induce cellular differentiation	*RARβ2*	6
Suppress apoptosis	*caspase 8; TMS-1*	7,8
Inhibit angiogenesis	*TSP-1*	9
DNA repair	*hMLH-1*	10
Hormonal transcription factors	*ERα*	11
Tumor-associated antigens	*MAGE*	12,13

Gene reactivation of indicated gene by 5-AZA-CdR was primarily demonstrated by experiments in cell culture using human tumor and leukemic cell lines. Aberrant methylation of these genes was demonstrated in primary tumors from patients using MSP or DNA sequencing.

*CDK, cyclin-dependent kinase; *RARbeta*, retinoic acid receptor beta; *TSP-1*, thrombospondin-1; *hMLH-1*, mismatch repair; *ERα*, estrogen receptor alpha.

drug in the cell. 5AZA-dCTP is a very good substrate for DNA polymerase and is rapidly incorporated into DNA.[22] Deamination of 5-AZA-CdR by cytidine deaminase results in a loss of its antineoplastic activity. The incorporation of 5-AZA-CdR into the 5-methylcytosine positions during the replication of DNA results in a potent inhibition of DNA methylation as a result of its irreversible inactivation of DNA methyltranferase due to the formation of a covalent bond between this enzyme and the 5-azacytosine ring (Fig 3).[23]

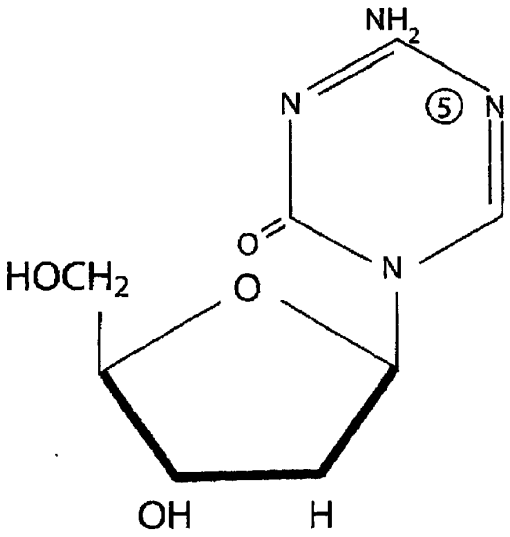

Figure 1 Chemical structure of 5-aza-2'-deoxycytidine (5-AZA-CdR). 5-AZA-CdRis an analog of deoxycytidine in which the carbon at position 5 of the pyrimidine ring is replaced by a nitrogen.

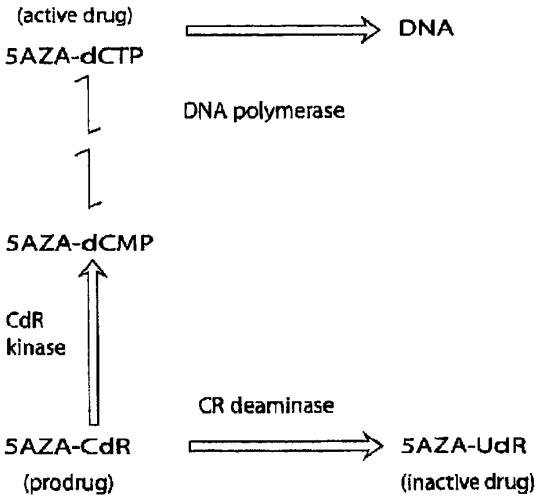

Figure 2 Metabolism of 5-aza-2'-deoxycytidine (5-AZA-CdR). CdR, deoxycytidine kinase; CR, cytidine.

Depletion of the DNA methyltransferase activity in the cell results in global hypomethylation of the genome and the reactivation of tumor-suppressor genes that were silenced by aberrant DNA methylation (Fig 4). The reactivation of the genes that suppress malignancy is responsible for the antineoplastic activity of 5-AZA-CdR. This gene reactivation can result in the induction of terminal differentiation[24] or senescence, growth inhibition, and loss of clonogenicity.[25,26]

Cellular Pharmacology

The major pharmacological characteristics of 5-AZA-CdR are summarized in Table 2. 5-AZA-CdR is an S-phase–specific agent. This means that this analog is only pharmacologically active in cells during the S phase of the cell cycle[27] In addi-

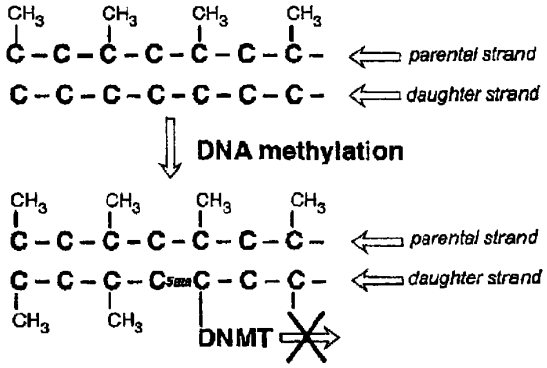

Figure 3 Molecular mechanism of action of 5-aza-2'-deoxycytidine (5-AZA-CdR). The incorporation of 5-AZA-CdR into DNA produces an inhibition of DNA methylation by the formation of a covalent bond between the 5-azacytosine ring (5azaC) and DNA methyltransferase (DNMT).

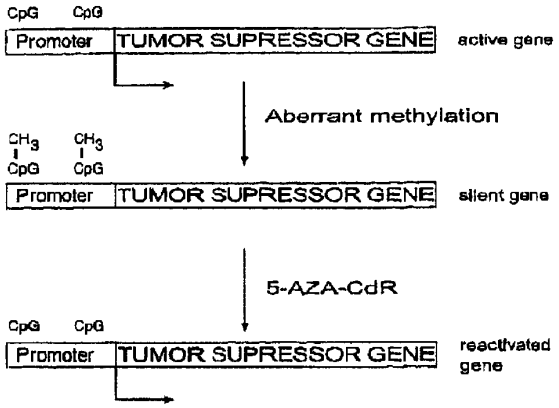

Figure 4 Reactivation of the expression of a silent tumor suppressor gene by demethylation of cytosine in the promoter region of the gene by 5-AZA-CdR.

tion, 5-AZA-CdR does not block the progression of cells from G1 phase into S phase. In many tumor cell lines after drug treatment there is a delay in the onset of the inhibition of growth and DNA synthesis. This type of inhibition becomes apparent only after two or three cell divisions. Using an in vitro clonogenic assay we observed that 5-AZA-CdR produced a loss of clonogenicity of all human leukemic and tumor cell lines tested, regardless of their phenotype. For most of these neoplastic cell lines, the IC_{90} (concentration that produces 90% response) of 5-AZA-CdR for the loss of clonogenicity is in the range of 100 ng/mL for a 24-hour drug exposure.[25,26] Longer drug exposures produced greater antineoplastic activity.

Drug Resistance

As summarized in Table 3, drug resistance to deoxycytidine analogs, such as 5-AZA-CdR, can occur by several different mechanisms.[28] Cells that are deficient in deoxycytidine kinase, the enzyme that activates 5-AZA-CdR, are completely resistant to this analog. Since cells contain two alleles for the deoxycytidine kinase gene, both alleles have to be inactivated for the cells to become completely resistant to 5-AZA-CdR, a rare event. Increased activity of cytidine deaminase, the enzyme that inactivates 5-AZA-CdR, produces drug resistance,[29] as does an increased intracellular pool of dCTP in neoplastic cells. The high level of dCTP will compete with 5AZA-dCTP and reduce its incorporation into DNA. Other possible mechanisms of resistance are a modified DNA methyltransferase that is not inactivated by the presence of 5-AZA-CdR in DNA and a modified DNA repair system that will remove 5-AZA-CdR from DNA. Currently, there are no experimental data to support these last two hypotheses.

Table 2 Pharmacological Characteristics of 5-AZA-CdR

- Prodrug activated by deoxycytidine kinase
- Inactivated by deamination with cytidine deaminase
- S-phase–specific agent
- Antineoplastic activity at low concentrations <20 ng/mL ($<10^{-6}$ mol/L)
- Excellent anatomical distribution (cerebral spinal fluid etc)
- Short in vivo half-life (15–20 min)
- High-dose therapy possible

Table 3 Mechanisms of Drug Resistance to 5-AZA-CdR

- Reduction or loss of deoxycytidine kinase
- Increase in cytidine deaminase
- Increase in intracellular pool of dCTP
- Increased DNA repair?
- Modification of DNMT activity?

Antineoplastic Activity in Animal Models

In mice with L1210 leukemia treated at the maximum tolerated dose (MTD) of each agent, 5-AZA-CdR was more potent than cytosine arabioside (ARA-C) and very much more potent than its related riboside analog, 5-azacytidine.[16] Under these experimental conditions, 5-AZA-CdR at the MTD produced "cures" of mice with L1210 leukemia, but ARA-C, one of the most active drugs for the therapy of acute myeloid leukemia in humans, did not.[30] The differences in the antileukemic potency of these two deoxycytidine analogs was not due to differences in their metabolism, since both analogs are metabolized by the same enzymes and incorporated into DNA. The major difference between the two analogs is their mechanism of action. ARA-C is a potent inhibitor of DNA polymerase, whereas 5-AZA-CdR is a potent inhibitor of DNA methyltransferase.

The antileukemic activity of 5-AZA-CdR in mice with L1210 leukemia increased with the dose and duration of the intravenous (IV) infusion. There was a very significant correlation between the antileukemic activity at different dose levels of 5-AZA-CdR and the extent of inhibition of global DNA methylation in the leukemic cells.[31] This observation supports the hypothesis that the antileukemic action of 5-AZA-CdR is related to the extent of its inhibition of DNA methylation. 5-AZA-CdR at a dose of 21.4 mg/kg administered as a 15-hour IV infusion showed curative potential in this leukemia model. Murine L1210 leukemic cells have an in vivo doubling time of about 12 hours. The estimated steady-state plasma level of 5-AZA-CdR in this experiment was about 1,000 ng/mL (~4 μmol/L).

We also evaluated the antitumor activity of 5-AZA-CdR in the mouse model with murine EMT6 mammary tumor.[32] The differentiation action of 5-AZA-CdR has the potential to convert a malignant tumor to a "benign" type of tumor, which will only slowly decrease in tumor size due to senescence. For this reason we designed an in vivo–in vitro model to obtain a more precise evaluation of the antineoplastic activity produced by 5-AZA-CdR against tumors . Following 5-AZA-CdR therapy of mice, the EMT6 tumor was excised, and the tumor cells disaggregated with trypsin and plated in petri dishes to measure survival by a colony assay. With this type of clonogenic assay we observed

that the antitumor activity of 5-AZA-CdR increased with the dose and duration of treatment. The "curative" dose-schedule in this tumor model was 30 mg/kg for an 18-hour infusion. The estimated steady-state plasma level of 5-AZA-CdR in this experiment was in the range of 1,000 ng/mL. The major toxicities produced by 5-AZA-CdR in animal models were leukopenia and intestinal ulceration.[33]

Clinical Studies on 5-AZA-CdR

Phase I and II Studies

A summary of the major clinical trials that have been performed on 5-AZA-CdR as a single agent on patients with hematologic malignancies and solid tumors is shown in Table 4. Several different dose-schedules have been investigated, producing a wide range of plasma concentrations of this analog. In patients with acute leukemia, dose-schedule A employed an intermediate dose of 5-AZA-CdR (1,000 to 2,000 mg/m^2 administered as a 36- to 60-hour infusion) producing plasma levels of this analog in the range of 150 to 200 ng/mL. This dose-schedule produced a response rate (complete [CR] and partial remission [PR]) of 35%. However, the responses were of short duration. Dose-schedule B (daily low-dose pulse for 10 days) produced a higher response rate (65%) and longer duration of remissions in patients with acute leukemia than did dose-schedule A.

In patients with chronic myeloid leukemia, dose-schedule C (low-dose [50 to 100 mg/m^2 administered as a 6-hour infusion every 8 hours for 3 days) produced a response rate of 43%, including some responses in patients with blastic crisis. In patients with myelodysplastic syndrome (MDS), dose-schedule D (low-dose [15 mg/m^2] administered as 4-hour infusion every 12 hours for 5 days) produced a 31% to 50% response rate. Dose-schedule E (low-dose [20 to 50 mg/m^2 administered as a 72-hour infusion) was also effective in patients with MDS, producing a response rate of 46%. However, this latter dose-schedule was ineffective in patients with different types of metastatic solid tumors.

In patients with different types of cancer dose-schedule F (25 to 100 mg/m^2 1 hour every 8 hours × 3) was ineffective. Dose-schedule G (200 to 660 mg/m^2 administered as an 8-hour infusion) showed some interesting responses in patients with non-small cell lung cancer (NSCLC). Six patients who received two or more cycles of this dose-schedule survived more than 15 months, including one patient who survived 81 months from the start of therapy.[20] After five cycles of 5-AZA-CdR, the latter patient was removed from the study due to signs of tumor progression. The patient then received a single treatment of vindesine. Although this unusual response may be considered anecdotal, this type of delayed action to 5-AZA-CdR is in accord with preclinical studies on this analog. In a second pilot study, two patients also showed very good responses to secondary chemotherapy after removal from the 5-AZA-CdR study (Soulières D, Momparler RL, unpublished data).

In conclusion, it is remarkable that different dose-schedules of 5-AZA-CdR showed responses in patients with hematologic malignancies. The clinical studies showed a wide range of plasma concentrations of 5-AZA-CdR at different durations (1 to 72 hours). At the dose-schedules investigated, 5-AZA-CdR showed more antineoplastic activity against the hematologic malignancies than against the solid tumors. However, the dose-schedules used in the treatment of hematologic malignancies did not result in any long-term survivors (>36 months), indicating that the optimal dose-schedule of 5-AZA-CdR still has to be found for this type of disease. Preliminary data on the therapy of solid tumors suggest that intense therapy with 5-AZA-CdR may be more effective than the low-dose therapy.

Hematopoietic Toxicity

The major toxicities produced by 5-AZA-CdR are granulocytopenia and thrombocytopenia.[18,19,45] For the intermediate

Table 4 Summary of Dose-Schedules Used in Clinical Trials on Decitabine as Single Agent in Patients With Different Types of Cancer

Type of Cancer	Dose (mg/m^2)	Schedule	Type of Dose-Schedule	Estimated Plasma Concentration (ng/mL)	References
ALL, AML	1,080–2,010	36–60 h	A	150–200	18,34
AML, CML, MDS	5–15	1 h × 10 d	B	30–120*	35
CML	50–100	6 h every 12 h × 5 d	C	50–100	36
MDS	15	4 h every 8 h × 3 d	D	25	37,38
MDS	50	72 h	E	15	39
HN	25–100	1 h every 8 h × 3	F	170–660*	40
Renal, col, HN, mel	75	1 h every 8 h × 3	F	500*	41
Prostate	75	1 h every 8 h × 3	F	500*	42
Testes	75	1 h every 8 h × 3	F	500*	43
Metastatic tumors	20–40	72 h	E	20	44
NSCLC	200–660	8 h	G	170–550	20,45

Response to 5-aza-2'-deoxycytidine treatment is summarized in text. The initial time in the schedule column is the duration of the infusion.
*Peak plama level during 1-hour infusion. Abbreviations: ALL, acute lymphoid leukemia; AML, acute myeloid leukemia; CML, chronic myeloid leukemia; MDS, myelodysplastic syndrome; HN, head and neck cancer; col, colorectal cancer; mel, melanoma; NSCLC, non-small cell lung cancer; inf, IV infusion.

doses of 5-AZA-CdR, the nadir for granulocytopenia occurs between days 14 and 28. Due to this leukopenia, a second cycle of 5-AZA-CdR should be administered 5 or 6 weeks after the initial treatment. The duration of thromobocytopenia is shorter than that of the granulocytopenia. An increase of the platelet count above normal range has been observed for some patients treated with 5-AZA-CdR. Since 5-AZA-CdR is an S-phase–specific agent, only the proliferating progenitor cells in the bone marrow are induced to undergo terminal differentiation, resulting in a depletion of the proliferating stem cells required to sustain normal hematopoiesis (Fig 5). However, the resting stem cells are not affected by the 5-AZA-CdR treatment, which permits the use of high-dose therapy with this analog. After therapy, the resting stem cells are recruited into the cell cycle and S phase to undergo proliferation. It is possible that the use of hematopoietic colony-stimulating factors after 5-AZA-CdR treatment may accelerate recovery from myelosuppression. The hematopoietic toxicity profile of 5-AZA-CdR is similar to its related deoxycytidine analog, ARA-C. Since high-dose therapy with ARA-C of 6 days duration can produce complete remissions in patients with acute leukemia, it may be possible to use 4 to 5 days therapy with 5-AZA-CdR in patients with a good hematologic status, without producing unacceptable hematopoietic toxicity.

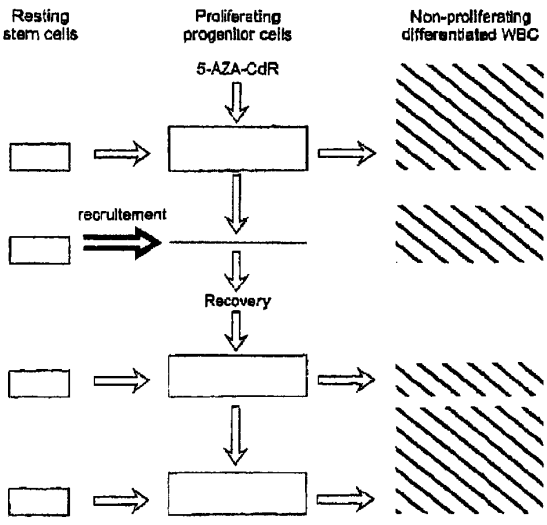

Figure 5 Hematopoietic toxicity produced by 5-AZA-CdR. Since 5-AZA-CdR is an S-phase–specific agent, its pharmacologic activity is directed primarily against proliferating progenitor cells. The resting (nonproliferating) stem cells in the bone marrow are completely insensitive to 5-AZA-CdR. After therapy these stem cells are recruited into the cells cycle to produce hematopoietic recovery. The use of hematopoietic growth factors after therapy has the potential to accelerate recruitment of the stem cells and reduce the duration of leucopenia and thromobocytopenia produced by 5-AZA-CdR.

Pharmacokinetics

The plasma beta half-life of 5-AZA-CdR is short, in the range of 15 to 25 minutes, due to rapid deamination by cytidine deaminase present in liver.[46] The presence of elevated levels of cytidine deaminase in this organ can provide a "biochemical sanctuary" for neoplastic cells, which can escape the cytotoxic action of 5-AZA-CdR, especially when low-dose treatment is used. There is excellent anatomical distribution of 5-AZA-CdR in the body fluids, including the cerebral spinal fluid. It has been reported that 5-AZA-CdR treatment alone cleared mennigeal leukemia.[34]

Optimization of Dose-Schedule of 5-AZA-CdR

Patient Selection

Since aberrant DNA methylation of genes that suppress malignancy has been detected in all types of malignancy, 5-AZA-CdR has the potential for therapy of all types of cancer. In support of this hypothesis are the preclinical investigations that have shown that all human leukemic and tumor cell lines lose their clonogenic potential upon in vitro treatment with this analog.[47] As for all anticancer drugs, the ideal time for treatment with 5-AZA-CdR is when the leukemic or tumor cell load is minimal.[48] Early diagnosis is very important. Ideally, patients should not be treated previously with genotoxic drugs, which have the potential to inactivate tumor-suppressor genes producing mutations and deletions. The genes in the damaged region of DNA cannot be reactivated by 5-AZA-CdR (Fig 6). Patients with a genetic predisposition to develop cancer may be interesting candidates for chemoprevention therapy with 5-AZA-CdR[49] using a conservative dose-schedule to minimize host toxicity. Patients with a poor hematologic status are candidates for only the low dose therapy of 5-AZA-CdR. However, if a response is obtained in these patients and their hematologic status improves, they may be candidates for intensive therapy with 5-AZA-CdR.

Dose Selection

One of the major reasons for failure to achieve curative chemothereapy with most genotoxic anticancer drugs, is that it is not possible to expose all the neoplastic cells in the different anatomical compartments to adequate cytotoxic levels of the drug due to the unacceptable host toxicity produced by high doses. Preclinical studies have shown that concentration of 5-AZA-CdR that produce a loss of clonogenicity (>90%) of both human leukemic and tumor cells are in the range of 100 ng/mL for a duration of 24 hours of drug exposure.[25,26,47] To achieve this level in the body fluids, 5-AZA-CdR should be infused at a rate of about 20 mg/m²/h.

Duration of Therapy

The ideal duration of therapy for 5-AZA-CdR should be long enough to permit all the neoplastic cells to enter the S phase of the cell cycle.[50] Since high-dose ARA-C of 144 hours duration can produce CRs of long duration in patients with

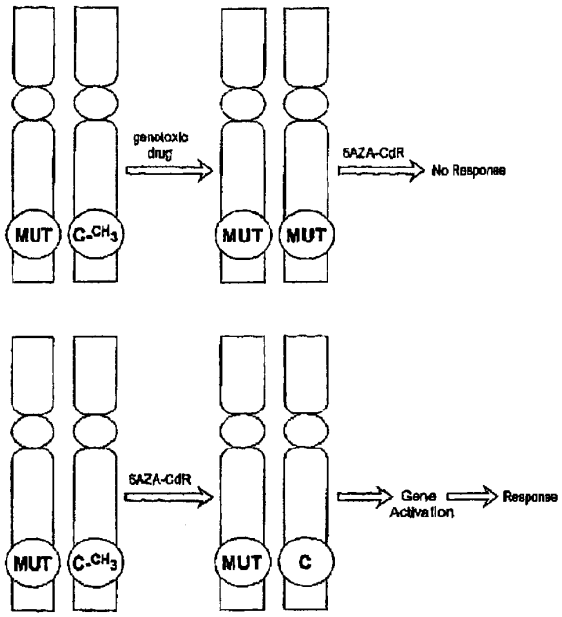

Figure 6 Both alleles of a tumor-suppressor gene must be inactivated to completely suppress its expression. In the illustration one allele is inactivated by mutation (MUT), whereas the second allele is inactivated by DNA methylation (C-CH_3). Therapy with a genotoxic anticancer drug has the potential to inactivate the second allele reducing the possibility of a response to subsequent therapy with 5-AZA-CdR by demethylation (C).

acute leukemia,[30] it suggests that during this interval most of the leukemic cells enter the S phase of the cell cycle. As mentioned earlier, since the hematopoietic toxicity produced by 5-AZA-CdR is similar to ARA-C, the duration of therapy of 72 to 96 hours with the 5-azacytosine analog may be possible without producing unacceptable hematopoietic toxicity in patients with leukemia.

The cell kinetics of most human tumors is different than that of leukemia because solid tumors in general have long cell cycle times. Therefore, the duration of chemotherapy for 5-AZA-CdR to achieve complete tumor eradication may have to be very long (>5 days), which will produce unacceptable hematopoietic toxicity. An interesting approach that merits investigation would be to use several cycles of intensive therapy with 5-AZA-CdR of short duration (12 to 24 hours). In this latter approach, the duration of the intensive chemotherapy with 5-AZA-CdR should last long enough to prevent significant tumor progression between cycles of therapy.

Evaluation of Response

The action of 5-AZA-CdR on neoplastic cells is often a delayed one, in which the cells undergo one or more cell divisions before gene reactivation and growth arrest occurs. This has been observed in both preclinical and clinical studies. In clinical studies patients with acute leukemia have been observed who underwent clinical remission although they showed a marrow with greater than 90% blasts at 28 days after 5-AZA-CdR treatment.[34]

In patients with solid tumors, due to the delayed action of 5-AZA-CdR, image analysis may show tumor progression and the patient may be removed from the study prematurely before several cycles of chemotherapy are completed. In this context, the molecular action of 5-AZA-CdR should be taken into consideration. A tentative guideline may be to not remove the patient from the study if tumor progression between cycles of 5-AZA-CdR is less than 20% as compared to the size of the tumor before the start of therapy. This latter approach will permit the use of several cycles of this analog and thus provide a greater opportunity to eradicate the proliferative potential of the tumor stem cells.

Discussion

To exploit the full chemotherapeutic potential of 5-AZA-CdR in cancer therapy, we have to find the optimal dose-schedule for this interesting inhibitor of DNA methylation. This requires knowledge of the pharmacology of this analog, its mechanism of action, its hematopoietic toxicity and the molecular targets in neoplastic cells. The novel mechanism of action of 5-AZA-CdR is very different from genotoxic anticancer drugs, suggesting that a different dose-schedule and different response analysis should be used for the deoxycytidine analog. The interesting responses obtained with 5-AZA-CdR in patients with hematologic diseases clearly indicate that it is an active chemotheapeutic agent. However, the dose-schedules used in these clinical studies did not produce long-term survivors, indicating that defining the optimal dose-schedule remains to be a key objective for future investigations. Careful analysis of the clinical data is required to determine the scientific rationale for both responses and relapses. Due to the heterogeneity and large number of target neoplastic cells, any weakness in the dose-schedule will permit some neoplastic cells to survive therapeutic intervention with 5-AZA-CdR. We have to determine why the chemotherapy with this analog fails to produce long-term responses in patients with cancer and to modify the dose-schedule accordingly.

In patients with acute leukemia, the probable reason for the failure of the dose-schedule A of 5-AZA-CdR (intensive therapy, 60 hours duration) to produce CRs of long duration (> 6 months) may have been due to the kinetics of the leukemic stem cells. The therapy of 60 hours duration may not have been long enough to permit all of the leukemic stem cells to enter the S phase. It should be noted that we detected signs of drug resistance in two patients with leukemia after treatment with 5-AZA-CdR.[31] One patient showed signs of deficiency in deoxycytidine kinase and the other showed increased levels of cytidine deaminase in blood leukemic blasts. Testing for drug resistance when patients with leukemia relapse can be used as an approach to find the optimal dose-schedule. The ideal dose-schedule of a drug should eradicate all of the drug-sensitive neoplastic cells

The reason that dose-schedule B (15 mg/m² for 10 days) did not produce many long-term survivors (>36 months)

was probably related to pharmacokinetics. The plasma concentration of 5-AZA-CdR with this latter regimen was so low that there was a high probability that some leukemic stem cells in the liver escaped the chemotherapeutic action of this analog, due to its rapid inactivation by the high levels of cytidine deaminase in this organ.

In clinical trials on 5-AZA-CdR in solid tumors the reason why dose-schedule F (intermediate dose every 8 hours × 3) was ineffective was probably due to both pharmacokinetic and cell kinetic reasons. The plasma concentration reached an intermediate level for only a short duration (<2 hours) due to the short half-life (15 to 25 minutes) of this analog in humans. In addition, the duration of therapy (~16 hours) was too short to permit a significant fraction of the tumor stem cells to enter S phase. It is interesting to note that this type of dose-schedule showed more clinical activity against patients with leukemia. The reason for this difference is not known, but may be due to higher levels of deoxycytidine kinase in leukemic cells as compared to tumor cells.

In patients with NSCLC, dose-schedule G of 5-AZA-CdR (~400 mg/m^2 administered as an 8-hour infusion) showed some interesting responses, but was ineffective in many patients due to cell kinetic reasons. The duration of the infusion (8 hours) was too short to permit a larger fraction of tumor cells to enter the S phase. Therefore, in some patients the tumors showed too much progression between cycles of 5-AZA-CdR therapy and these patients were removed from the study. As mentioned earlier, the longest survivor (81 months) received five cycles of 5-AZA-CdR. Due to some signs of tumor progression this patient was removed form the study and received a single course of therapy with vindesine. This response is a good example of the delayed action of 5-AZA-CdR and it raises the question: does prior treatment with 5-AZA-CdR make the tumor cells more sensitive to the antineoplastic action of other drugs? Preclinical studies support this hypothesis.

5-AZA-CdR treatment of tumor cells in which the expression of the human mismatch DNA repair gene (*hMLH1*) was silenced by aberrant DNA methylation reactivated the expression of this DNA repair gene and made the tumor cells more sensitive to the cytotoxicity induced by cisplatin.[52] Other possible explanations for the increased sensitivity of tumor cells to anticancer drugs following pretreatment with 5-AZA-CdR are the reactivation of the genes for apoptosis or senescence in the target tumor cells by this analog.

Immunologic events may have some potential to affect the antineoplastic activity of 5-AZA-CdR. This analog was reported to re-activate the expression of tumor-associated antigens (MAGE, NY-ESO) in tumor cells.[12,13,53] In addition, 5-AZA-CdR was also reported to upregulate the expression of major histocompatibility complex (MHC)-I in tumor cells, which can enhance their recognition by cytotoxic T cells.[54] Both of these events may stimulate T lymphocytes to target the tumor cells. Future investigations will clarify the role of the immune system in the response to 5-AZA-CdR therapy.

The use of 5-AZA-CdR in combination with other agents that have the potential to enhance its antineoplastic activity also merits investigation. A "cross-talk" takes place between DNA methylation and histone deacetylation of chromatin at the promoter region of genes to silence gene expression.[14] This is due to the attachment of 5-methylcytosine-binding proteins to the methylated promoter and its recruitment of histone deacetylase, which enhances the suppression of transcription by conversion of chromatin from an open to a compact configuration.

These two epigenetic events can be reversed by using 5-AZA-CdR in combination with an inhibitor of histone deacetylase. Preclinical studies have shown that the interaction between these two classes of agents produces a synergistic antineoplastic effect and a synergistic reactivation of tumor-suppressor genes on both leukemic and tumor cells.[25,26,55–58] Histone deacetylase inhibitors show antineoplastic activity as single agents and are currently under clinical investigation.

Another interesting agent to use in combination with 5-AZA-CdR in cancer therapy is zebularine, a potent inhibitor of the deamination of the 5-azacytosine analog by cytidine deaminase.[59] zebularine is also a weak, but significant, inhibitor of DNA methylation and shows antineoplastic activity as a single agent.[60] This dual action makes zebularine a very interesting agent to use in combination with 5-AZA-CdR. In mice with L1210 leukemia this inhibitor of cytidine deaminase was shown to enhance the antineoplastic action of 5-AZA-CdR and the reactivation of the p57 tumor-suppressor gene in this leukemic cell line.[61] Zebularine has the potential to eradicate leukemic cells that use the liver as a "biochemical sanctuary" due to its high levels of cytidine deaminase. These leukemic cells can escape the antineoplastic action of 5-AZA-CdR. Biochemical modulators, which reduce the intracellular pool size of dCTP and its competition with 5-AZA-CdR for incorporation into DNA, also have been shown to enhance the antineoplastic activity of 5-AZA-CdR against leukemic cells.[48]

Although these other agents are very interesting, the first priority should be to find the optimal dose-schedule for 5-AZA-CdR as a single agent. Otherwise it is possible to end up with a clinical protocol that uses a suboptimal dose-schedule for 5-AZA-CdR in combination with a second chemotherapeutic agent.

Conclusions

The epigenetic silencing of many genes that suppress transformation to a malignant phenotype by aberrant DNA methylation provides interesting targets for chemotherapeutic intervention. Since this epigenetic change is reversible, the potent and specific inhibitor of DNA methylation, 5-AZA-CdR, merits clinical investigation for cancer therapy. This analog was shown to reactivate many types of tumor-suppressor genes in human tumor and leukemic cell lines. In animal models, 5-AZA-CdR was demonstrated to be a very potent antineoplastic agent against both leukemia and tumors. In clinical trials, 5-AZA-CdR has shown promising activity against hematologic malignancies and lung cancer. Since this analog is an S-phase–specific agent with a short in vivo half-life, its antineoplastic activity is highly schedule-

dependent. The initial primary objective should be to find the optimal dose-schedule for 5-AZA-CdR. This aim requires a sound understanding of the pharmacology of this analog, its novel mechanism of action, tumor cell biology, and the cell kinetics of normal and neoplastic tissue. The collaboration between laboratory investigators and clinicians is essential. Using this approach, it should be possible in the near future to exploit the full chemotherapeutic potential of 5-AZA-CdR for the therapy of cancer.

References

1. Jones PA, Laird PW: Cancer epigenetics comes of age. Nature Genet 21:163-167, 1999
2. Momparler RL: Cancer epigenetics. Oncogene 22:6479-6483, 2003
3. Herman JG, Jen J, Merlo A, et al: Hypermethylation-associated inactivation indicates a tumor suppressor role for p15INK4B1. Cancer Res 56:722-727, 1996
4. Otterson GA, Khleif SN, Chen W, et al: CDKN2 gene silencing in lung cancer by DNA hypermethylation and kinetics of p16INK4 protein induction by 5-aza-2'-deoxycytidine. Oncogene 11:1211-1216, 1995
5. Graff JR, Herman JG, Lapidus RL, et al: E-cadherin expression is silenced by DNA hypermethylation in human breast and prostate carcinomas. Cancer Res 55:5195-5199, 1995
6. Côté S, Sinnett D, Momparler RL: Demethylation by 5'-aza-2'-deoxycytidine of specific 5-methylcytosine sites in the promoter region of the retinoic acid receptor beta gene in human colon carcinoma cells. Anticancer Drugs 9:743-750, 1998
7. Teitz T, Wei T, Valentine MB, et al: Caspase 8 is deleted or silenced preferentially in childhood neuroblastomas with amplification of MYCN. Nature Med 6:529-535, 2000
8. Conway KE, McConnell BB, Bowring CE, et al: TMS1, a novel proapoptotic caspase domain protein, is a target of methylation-induced silencing in human breast cancer. Cancer Res 60:6236-6242, 2000
9. Li Q, Ahuja N, Burger PC, et al: Methylation and silencing of the thrombospondin-1 promoter in human cancer. Oncogene 18:3284-3289, 1999
10. Herman JG, Umar A, Polyak K, et al: Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. Proc Natl Acad Sci U S A 95:6870-6875, 1998
11. Ferguson AT, Lapidus RG, Baylin SB, et al: Demethylation of the estrogen receptor gene in estrogen receptor-negative breast cancer cells can reactivate estrogen receptor gene expression. Cancer Res 55:2279-2283, 1995
12. Weber J, Salgaller M, Samid D, et al: Expression of the MAGE-1 tumor antigen is up-regulated by the demethylating agent 5-aza-2'-deoxycytidine. Cancer Res 54:1766-1771, 1994
13. Sigalotti L, Corsi S, Nardi G, et al: Promoter methylation controls the expression of MAGE 2, 3 and 4 genes in human cutaneous melanoma. J Immunother 25:16-26, 2002
14. Jones PA, Veestra GJC, Wade PA, et al: Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. Nature Genet 19: 187-191, 1998
15. Momparler RL, Bovenzi V: DNA methylation and cancer. J Cell Physiol 183:145-154, 2000
16. Momparler RL, Momparler LF, Samson J: Comparison of the antileukemic activity of 5-aza-2'-deoxycytidine, 1-β-D-arabinofuranosyl-cytosine and 5-azacytidine against L1210 leukemia. Leuk Res 8:1043-1049, 1984
17. Braakhuis BJM, van Dongen GAMS, van Walsum M, et al: Preclinical antitumor activity of 5-aza-2'-deoxycytidine against human head and neck cancer xenografts. Invest New Drugs 6:299-304, 1988
18. Momparler RL, Rivard GE, Gyger M: Clinical trial on 5-aza-2-deoxycytidine in patients with acute leukemia. Pharmac Ther 30:277-286, 1986
19. Issa JP: Decitabine. Curr Opin Oncol 15:446-451, 2003
20. Momparler RL, Ayoub J: Potential of 5-aza-2'-deoxycytidine (decitabine) a potent inhibitor of DNA methylation for therapy of advanced non-small cell lung cancer. Lung Cancer 34:S111-S115, 2001
21. Momparler RL: Molecular, cellular and animal pharmacology of 5-aza-2-deoxycytidine. Pharmac Ther 30:287-299, 1986
22. Bouchard J, Momparler RL: Incorporation of 5-aza-2'-deoxycytidine 5'-triphosphate into DNA: Interactions with mammalian DNA polymerase and DNA methylase. Mol Pharmacol 24:109-114, 1983
23. Jüttermann R, Li E, Jaenisch R: Toxicity of 5-azn-2'-deoxycytidine to mammalian cells is mediated primarily by covalent trapping of DNA methyltransferase rather than DNA demethylation. Proc Natl Acad Sci U S A 91:11797-11801, 1994
24. Jones PA, Taylor SM: Cellular differentiation, cytidine analogs and DNA methylation. Cell 20:85-93, 1980
25. Bovenzi, V, Momparler RL: Antineoplastic action and expression of retinoic acid receptor β gene in breast carcinoma cells treated with 5-aza-2'-deoxycytidine and an histone deacetylase inhibitor. Cancer Chemother Pharmacol 48:71-76, 2001
26. Primeau M, Gagnon J, Momparler RL: Synergistic antineoplastic action of DNA methylation inhibitor 5-aza-2'-deoxycytidine and histone deacetylase inhibitor depsipeptide on human breast carcinoma cells. Int J Cancer 103:177-184, 2003
27. Momparler RL, Samson J, Momparler LF, et al: Cell cycle effects and cellular pharmacology of 5-aza-2'-deoxycytidine. Cancer Chemother Pharmacol 13:191-194, 1984
28. Momparler RL, Onetto-Pothier N: Drug resistance to cytosine arabinoside, in Kessel D (ed): Resistance to Antineoplastic Drugs. Boca Raton, FL, CRC Press, 1988, pp 353-367
29. Eliopoulos N, Momparler RL: Drug resistance to 5'-aza-2'-deoxycytidine, 2,2'-difluorodeoxycytidine and cytosine arabinoside conferred by retroviral-mediated transfer of human cytidine deaminase cDNA into murine cells. Cancer Chemother Pharmacol 42:373-378, 1998
30. Weick JK, Kopecky KJ, Applebaum FR, et al: A randomized investigation of high-dose versus standard dose cytosine arabinoside with daunomycin in patients with previously untreated acute myeloid leukemia: A Southwest Oncology Group study. Blood 88:2841-2851, 1996
31. Wilson VL, Jones PA, Momparler RL: Inhibition of DNA methylation in L1210 leukemic cells by 5-aza-2'-deoxycytidine as a possible mechanism for chemotherapeutic action. Cancer Res 43:349-3497, 1983
32. Chabot G: Pharmacocinétique et effets antinéoplasiques de al 5-aza-2'-desoxycytidine chez les animaux. PhD thesis, Université de Montréal, 1983, pp 166-174
33. Momparler RL, Frith CH: Toxicology in mice of the antileukemic agent 5-aza-2'-deoxycytidine. Drug Chem Toxicol 4:373-381, 1981
34. Rivard GE, Momparler RL, Demers J, et al: Phase I study on 5-aza-2'-deoxycytidine in children with acute leukemia. Leuk Res 5:453-462, 1981
35. Issa JP, Garcia-Manero G, Giles FJ, et al: Phase 1 study of low-dose prolonged exposure schedules of the hypomethylating agent 5-aza-2'-deoxycytidine (decitabine) in hematopoietic malignancies. Blood 103: 1635-1640, 2004
36. Kantarjian HM, O'Brien SM, Cortes J, et al: Results of decitabine (5-aza-2'-deoxycytidine) therapy in 130 patients withchronic myelogenous leukemia. Caner 98:522-528, 2003
37. Zagonel V, Lo Re G, Marotta G, et al: 5-Aza-2'-deoxycytidine (decitabine) induces trilineage response in unfavorable myelodysplastic syndromes. Leukemia 7:30-35, 1993 (suppl 1)
38. Lübbert M, Wijermans P, Kunzmann R et al: Cytogentic responses in high-risk myelodysplastic syndrome following low-dose treatment with the DNA methylation inhibitor 5-aza-2'-deoxycytidine. Br J Haematol 114:349-357, 2001
39. Wijermans PW, Krulder JWM, Huijgens PC: Continuous infusion of low-dose 5-aza-2'-deoxycytidine in elderly patients with high risk myelodysplastic syndrome. Leukemia 11:1-3, 1997
40. van Groeningen CJ, Leyva A, O'Brien AM, et al: Phase I and pharmacokinetic study on 5-aza-2'-deoxycytidine (NSC 127716) in cancer patients. Cancer Res 46:4831-4836, 1986
41. Abele R, Clavel M, Dodion P et al: The EORTC Early Clinical Trials Cooperative Group experience with 5-aza-2'-deoxycytidine (NSC

127716) in patients with colo-rectal, head and neck, renal carcinomas and malignant melanomas. Eur J Clin Oncol 23:1921-1924, 1987
42. Thibault A, Figg WD, Bergan RC, et al: A phase II study of 5-aza-2′-deoxycytidine (decitabine) in hormone independent metastatic (D2) prostate cancer. Tumori 84:87-89, 1998
43. Clavel M, Monfardini S, Fossa S, et al: 5-Aza-2′-deoxycytidine (NSC 127716) in non-seminomatous testicular cancer. Phase II from EORTC Early Clinical Trials Cooperative Group and Genito-urinary Group. Ann Oncol 3:399-400, 1992
44. Aparicio A, Eads CA, Leong LA, et al: Phase I trial of continuous infusion of 5-aza-2′-deoxycytidine. Caner Chemother Pharmacol 51:231-239, 2003
45. Momparler RL, Bouffard DY, Momparler LF, et al: Pilot phase I-II study on 5-aza-2′-deoxycytidine (decitabine) in patients with metastatic lung cancer. Anticancer Drugs 8:358-368, 1997
46. Ho DHW: Distribution of kinase and deaminase of 1-β arabinofuranosylcytosine in tissues of man and mouse. Cancer Res 33:2816-2820, 1973
47. Momparler RL, Onetto-Pothier N, Momparler LF: Comparison of antineoplastic activity of cytosine arabinoside and 5-aza-2′-deoxycytidine against human leukemic cells of different phenotype. Leuk Res 14:755-760, 1990
48. Momparler RL, Côté, S, Eliopoulos N: Pharmacological approach for optimization of the dose-schedule of 5-aza-2′-deoxycytidine (decitabine) for the therapy of leukemia. Leukemia 11:175-180, 1997
49. Laird PW, Jackson-Grusby L, Fazeli A, et al: Suppression of intestinal neoplasia by DNA hypomethylation. Cell 81:197-205, 1995
50. Momparler RL: Model for the chemotherapy of acute leukemia with 1-β-arabinofuranosylcytosine. Cancer Res 34:1775-1787, 1974
51. Onetto N, Momparler RL, Momparler LF, et al: In vitro tests to evaluate the response to therapy of acute leukemia with cytosine arabinoside or 5-aza-deoxycytidine. Semin Oncol 14:231-237, 1987
52. Plumb JA, Sthrathdee G, Sludden J et al: Reversal of drug resistance in human tumor xenografts by 2′-deoxy-5-azacytidine-induced demethylation of the hMLH1 gene promoter. Cancer Res 60:6039-6044, 2000
53. Weiser TS, Sheng Guo Z, Ohnmacht GA, et al: Sequential 5-aza-2 deoxycytidine-depsipeptide FR901228 treatment induces apoptosis preferentially in cancer cells and facilitates their recognition by cytolytic T lymphocytes specific for NY-ESO-1. J Immunother 24:151-161, 2001
54. Coral S, Sigalotti L, Gasparollo A, et al: Prolonged regulation of the expression of HLA class I antigens and costimulatory molecules on melanoma cells treated with 5-aza-2′-deoxycytidine (5-AZA-CdR). J Immunother 22:16-24, 1999
55. Cameron EE, Bachman KE, Myöhänen S, et al: Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. Nature Genet 21:103-107, 1999
56. Shaker S, Bernstein M, Momparler RL: Antineoplastic action of 5-aza-2′-deoxycytidine (dacogen) and depsipeptide on Raji lymphoma cells. Oncol Rep 11:1253-1256, 2004
57. Lemaire M, Momparler LF, Farinha NJ, et al: Enhancement of antineoplastic activity of 5-aza-2′-deoxycytidine byphenylbutyrate on L1210 leukemic cells. Leuk Lymphoma 45:147-154, 2004
58. Boivin A-J, Momparler LF, Hurtubise A., et al: Antineoplastic action of 5-aza-2′-deoxycytidine and phenylbutyrate on human lung cancer. Anticancer Drugs 13:1-6, 2002
59. Laliberté J, Marquez VE, Momparler RL : Potent inhibitors for the deamination of cytosine arabinoside and 5-aza-2′-deoxycytidine by human cytidine deaminase. Cancer Chemother Pharmacol 30:7-11, 1992
60. Cheng JC, Matsen CB, Gonzales FA, et al: Inhibition of DNA methylation and reactivation of silenced genes by zebularine. J Natl Cancer Inst 95:399-409, 2003
61. Lemaire M, Momparler LF, Bernstein M, et al: Enhancement of antineoplastic action of 5-aza-2′-deoxycytidine by zebularine on L1210 leukemia. Anticancer Drugs 16:301-308, 2005

Published OnlineFirst January 28, 2011; DOI:10.1158/1078-0432.CCR-10-1893

Clinical Cancer Research

Phase I Study of Decitabine in Combination with Vorinostat in Patients with Advanced Solid Tumors and Non-Hodgkin's Lymphomas

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Abstract

Purpose: This phase I study evaluated the safety, tolerability, pharmacokinetics, and preliminary efficacy of the combination of decitabine with vorinostat.

Patients and Methods: Patients with advanced solid tumors or non-Hodgkin's lymphomas were eligible. Sequential and concurrent schedules were studied.

Results: Forty-three patients were studied in 9 different dose levels (6 sequential and 3 concurrent). The maximum tolerated dose (MTD) on the sequential schedule was decitabine 10 mg/m^2/day on days 1 to 5 and vorinostat 200 mg three times a day on days 6 to 12. The MTD on the concurrent schedule was decitabine 10 mg/m^2/day on days 1 to 5 with vorinostat 200 mg twice a day on days 3 to 9. However, the sequential schedule of decitabine 10 mg/m^2/day on days 1 to 5 and vorinostat 200 mg twice a day on days 6 to 12 was more deliverable than both MTDs with fewer delays on repeated dosing and it represents the recommended phase II (RP2D) dose of this combination. Dose-limiting toxicities during the first cycle consisted of myelosuppression, constitutional and gastrointestinal symptoms and occurred in 12 of 42 (29%) patients evaluable for toxicity. The most common grade 3 or higher adverse events were neutropenia (49% of patients), thrombocytopenia (16%), fatigue (16%), lymphopenia (14%), and febrile neutropenia (7%). Disease stabilization for 4 cycles or more was observed in 11 of 38 (29%) evaluable patients.

Conclusion: The combination of decitabine with vorinostat is tolerable on both concurrent and sequential schedules in previously treated patients with advanced solid tumors or non-Hodgkin's lymphomas. The sequential schedule was easier to deliver. The combination showed activity with prolonged disease stabilization in different tumor types. *Clin Cancer Res; 17(6); 1582–90. ©2011 AACR.*

Introduction

Hypermethylation of cytosines in CpG dinucleotides in the promoter regions of tumor-suppression genes and deacetylation of amino acid residues on the histone tails of nucleosomes, represent 2 epigenetic mechanisms of gene silencing that can contribute to tumor formation and progression (1, 2). Both events are considered reversible, and agents that inhibit the enzymes responsible for DNA methylation and histone deacetylation have been developed as anticancer agents (3).

Decitabine (5-aza-2′-deoxycytidine), a nucleoside analogue that is incorporated into DNA and acts as an hypomethylating agent by inhibiting DNA methyltransferase, and vorinostat (suberoylanilide hydroxamic acid), a small molecule that binds and directly inhibits histone deacetylase, are 2 agents with epigenetic effects that have shown clinical antitumor activity and are now approved for the treatment of myelodysplastic syndrome and cutaneous T-cell lymphoma, respectively (4–7). The validation of epigenetic treatments as anticancer strategies has supported an increasing number of trials evaluating epigenetic agents alone or in combination with other agents in both hematologic and solid malignancies (8, 9).

The combination of DNA methyltransferase inhibitor (DNMTi) with a histone deacetylase inhibitor (HDACi) represents an area that is gaining attention in the clinical development of epigenetic therapies. This concept is supported by preclinical evidence that DNA methylation and histone deacetylation are functionally linked, leading to transcriptional inactivation of genes critical for tumorigenesis (10, 11). Moreover, the *in vitro* combination of a DNMTi with an HDACi in hematologic and solid tumor

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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doi: 10.1158/1078-0432.CCR-10-1893

Published OnlineFirst January 28, 2011; DOI:10.1158/1078-0432.CCR-10-1893

Translational Relevance

Hypermethylation of cytosines in CpG dinucleotides in the promoter regions of tumor suppression genes and deacetylation of amino acid residues on the histone tails of nucleosomes, represent two epigenetic mechanisms of gene silencing that can contribute to tumor formation and progression. This phase I–targeted combination trial evaluates the safety, tolerability, pharmacokinetics, and preliminary antitumor activity of the hypomethylating agent decitabine, plus the histone deacetylase inhibitor vorinostat, in patients with advanced solid tumors. Sequential and concurrent administration schedules of these two agents were studied. Dose-limiting toxicities consisted mainly of myelosuppression, constitutional and gastrointestinal symptoms. Disease stabilization for four or more cycles was observed in about 30% of patients. Although the combination of these two types of epigenetics-modulating agents has been examined in hematological malignancies, this study represents one of the first attempts of this strategy in advanced solid tumors.

cell lines have shown synergistic effects resulting in increased gene reexpression and superior antitumor activity (12–14).

The optimal schedule of the combination of a DNMTi with an HDACi has not been established yet. Although most of the preclinical studies performed have used a sequential administration of DNMTi followed by HDACi, it remains unclear whether different schedules of administration may have better clinical activity. In the phase I trial reported here, the combination decitabine and vorinostat was studied for the first time in patients with solid tumors and non-Hodgkin's lymphomas (NHL). Two different schedules of administration, sequential and concurrent, were evaluated. The principal objective of this study was to determine the safety and tolerability of the combination. Secondary objectives included the assessments of pharmacokinetics (PK) and preliminary antitumor efficacy.

Patients and Methods

Patient selection

Patients were eligible if they had a histologically or cytologically documented advanced solid malignancy or NHL, refractory to standard therapy or for which no standard therapy existed. Other key eligibility criteria included: Eastern Cooperative Oncology Group performance status 0 to 2; adequate hematologic, hepatic, and renal functions [white blood cell count $\geq 3 \times 10^9$/L, absolute neutrophil count (ANC) $\geq 1.5 \times 10^9$/L, platelets $\geq 100 \times 10^9$/L, AST (aspartate aminotransferase)/ALT (alanine aminotransferase) $\leq$ 2.5 times upper limit of normal, bilirubin within normal limits, creatinine $\leq$ 150 mmol/L and creatinine clearance $\geq$ 60 mL/minute]; unlimited prior chemotherapy, radiotherapy or targeted agents with at least 3-week interval (6-week interval if prior nitrosoureas or mitomycin C) between study entry and any prior treatment; no valproic acid or other HDACi for at least 2 weeks before study entry; no prior decitabine; and no known brain metastases.

The institutional review board of both participating centers approved the study, which was conducted in accordance with federal and institutional guidelines.

Study design and patient evaluation

This was a 2-center, open-label, phase I study in which intravenous decitabine administered on days 1 to 5 was combined with oral vorinostat either in a sequential (vorinostat starting on day 6) or a concurrent schedule (vorinostat starting on day 3), in 28-day cycles. The study began with dose escalation on the sequential schedule, and once the maximum tolerated dose (MTD) was established, accrual began on the concurrent schedule. On the sequential schedule, the starting dose of decitabine was 20 mg/m^2/day on days 1 to 5, given as an intravenous infusion over 1 hour. Vorinostat was given at a starting dose of 100 mg twice a day on days 6 to 21. The starting dose of decitabine was chosen on the basis of published data showing good tolerability and higher response rates in patients with myelodysplastic syndrome treated with 20 mg/m^2/day for 5 days (15). The starting dose of vorinostat was based on previous monotherapy studies showing that the maximum tolerated dose was 200 mg orally twice a day continuously in patients with solid tumors and 250 mg orally thrice a day for 14 days every 21 days in patients with hematologic malignancies (16, 17). No dose escalation or modification of the duration of treatment with decitabine was planned, whereas both dose escalation (up to 200 mg orally thrice a day) and evaluation of different durations of treatment (7, 16, or 21 days, starting on day 6) were initially planned for vorinostat. For the concurrent schedule, the starting doses of decitabine and vorinostat were based on the MTD established on the sequential schedule and dose escalation and evaluations of different durations of treatment (7 or 14 days, starting on day 3) were planned for vorinostat only.

Dose escalation in both schedules followed the standard 3 + 3 rule. The RP2D of this study was defined as the dose level at which 1 or less than 1 of 6 patients developed dose-limiting toxicity (DLT) and had the lowest frequency of treatment delays. Toxicity was graded using the National Cancer Institute Common Terminology Criteria for Adverse Events version 3.0. DLTs were defined as adverse events occurring during the first cycle of treatment and fulfilling one of the following criteria: ANC $< 0.5 \times 10^9$/L for 7 days or more, febrile neutropenia, platelets less than 25×10^9/L or grade 3 thrombocytopenia associated with bleeding; any grade 3 or higher or intolerable grade 2 nonhematologic toxicity except alopecia, nausea, and vomiting responsive to antiemetics, diarrhea responsive to medications, and electrolyte abnormalities correctable with supportive therapy; and any toxicity resulting in treatment delay of more than 2 weeks. Response was assessed every 2 cycles using the Response Evaluation Criteria in Solid Tumors (18).

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Pretreatment evaluation and safety assessment

Pretreatment evaluation consisted of a complete medical history, physical examination, vital signs, electrocardiogram, complete blood count (CBC), serum chemistries, prothrombin time/INR, and activated partial thromboplastin time, serum or urine pregnancy test and baseline tumor measurements. On days 1, 8, and 15 of each cycle, evaluation consisted of a brief history and physical examination (day 1), vital signs (day 1), CBC, and serum chemistries.

Dose modification

Patients who experienced any DLT had treatment delayed by 1-week intervals until recovery and then may have continued on study with reduction of decitabine and vorinostat by one dose level. If no recovery occurred after a delay of 3 weeks of the next scheduled treatment cycle, patients were discontinued from protocol treatment. Blood counts must be recovered to begin a new treatment cycle.

Duration of study treatment

Patients with an objective response or stable disease were allowed to remain on study until disease progression. Otherwise, study treatment continued until disease progression, unacceptable adverse event, patient's decision to withdraw consent, or changes in the patient's condition that rendered the continuation of study treatment unacceptable.

Pharmacokinetic analysis

Blood samples for evaluation of decitabine were collected on days 1 and 5 of cycle 1, before dosing, 30 minutes after the infusion had started, at the end of infusion and at 5, 20, 35, 45, and 60 minutes from the end of infusion. Blood was collected into a sodium heparin Vacutainer tube and centrifuged at 1,500 × *g* for 15 minutes. The resulting plasma was transferred into polypropylene tubes and stored at −70°C until analyzed for decitabine concentration, using a validated high performance liquid chromatography with tandem mass spectrometry (LC-MS/MS) (19). On the sequential schedule, blood samples for vorinostat were collected on day 9 of cycle 1 before dosing and at 0.5, 1, 2, 2.5, 3, 4, 6, and 8 hours after dosing. On the concurrent schedule, blood samples were collected on day 4 of cycle 1 at the same time points. Samples were allowed to clot at 4°C for 20 to 30 minutes, and then centrifuged at 2,000 × *g* for 15 minutes at 4°C. The resulting serum was transferred to polypropylene cryotubes and stored at −70°C until analyzed for vorinostat concentrations with a validated LC-MS/MS assay (20). PK parameters were calculated by noncompartmental methods using WinNonlin (Version 5.2; Pharsight Corp.)

Exploratory analyses were performed for PK parameters and adjustments made for multiple comparisons. *t* Tests were used for independent group comparisons and paired *t* tests were used to compare PK parameters of decitabine on day 1 versus day 5 within a dose level.

Results

Patient characteristics

Forty-four patients were enrolled into this study and 43 received treatment for a total of 136 cycles (median 2, range: 1–25; Table 1). One patient with neuroendocrine carcinoma of the pancreas did not receive treatment because baseline CT scans showed no significant growth of disease.

Table 1. Patient characteristics

Characteristic	Patients	
	n	%
No of patients	44	
Median age, y (Range)	62 (31–77)	
Sex		
Female	18	40
Male	26	60
ECOG PS		
0	12	27
1	29	66
2	3	7
Primary tumor type		
Colorectal	11	25
NHL	4	9
Breast	3	7
Melanoma	3	7
Cholangiocarcinoma	2	5
NSCLC	2	5
Duodenal	1	2
Mesothelioma	1	2
Appendix	1	2
Uterine (adenocarcinoma and sarcoma)	2	5
Stomach	1	2
Pancreas (mucinous cystic carcinoma and neuroendocrine)	2	5
Fallopian tube	1	2
Thymus	1	2
Liver	1	2
Ovarian (Sertoli-Leyding cell)	1	2
Parotid (Non–small-cell and adenoid cystic)	2	5
Sweat gland adenocarcinoma	1	2
Lacrimal gland (adenoid cystic)	1	2
Submandibular gland (adenoid cystic)	1	2
Nasal cavity (adenoid cystic)	1	2
Oral cavity (adenoid cystic)	1	2
No. of prior systemic treatments		
0	3	7
1	8	18
2	9	20
3	10	23
≥4	14	32

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All patients had progressed from previous treatments for advanced disease. The median number of prior systemic treatment lines was 3. Three patients were treated previously with radiation therapy only and had not received systemic therapy.

A total of 43 patients received at least 1 cycle of study treatment. At the time of data cut off (March 2010), treatment was discontinued due to radiologically confirmed progression or due to symptomatic deterioration caused by underlying disease in 37 patients; 5 patients discontinued due to adverse events related to study treatment; 1 patient with stable disease after 25 cycles remains on study. There were no treatment-related deaths.

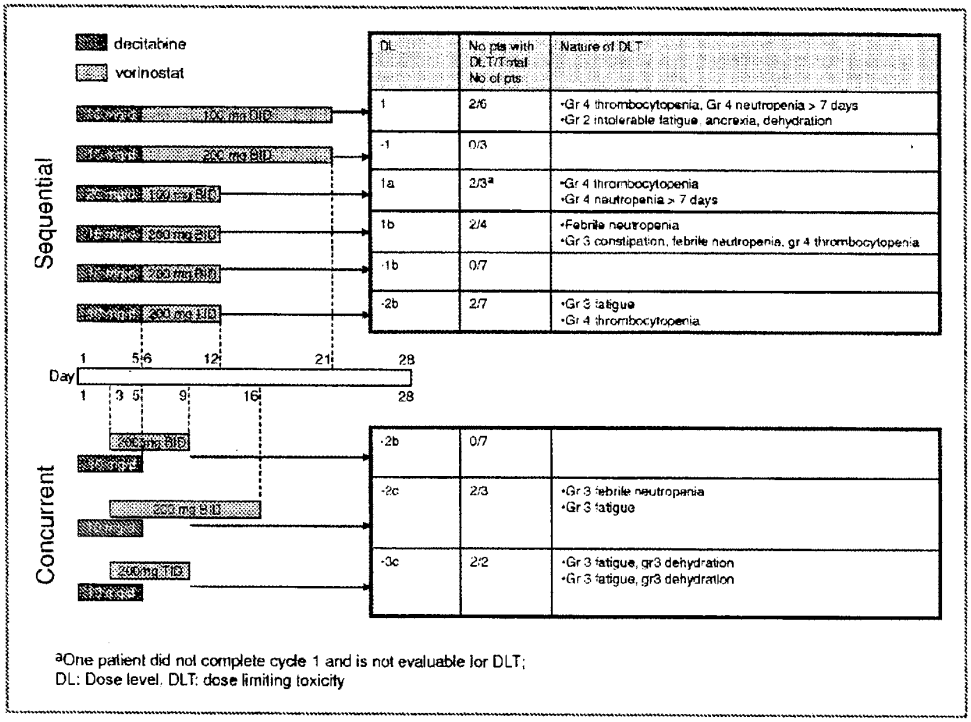
Dose escalation and dose-limiting toxicities

Six dose levels were evaluated on the sequential and 3 on the concurrent schedule (Fig. 1). On the sequential schedule, 8 of 30 (27%) evaluable patients experienced at least 1 DLT. The nature of DLTs was as follows: hematologic in 5 patients, nonhematologic in 2, and both hematologic and nonhematologic in 1 patient. Among hematologic DLTs, grade 4 thrombocytopenia occurred in 4 patients, grade 4 neutropenia lasting 7 days or more occurred in 2 and febrile neutropenia occurred in 2 patients. There was a clear association between hematologic DLTs and higher doses of decitabine. In fact, among 14 patients treated at the 2 dose levels with decitabine given at 10 mg/m^2/day, only 1 heavily pretreated patient with NHL who had a prior autotransplant developed a hematologic DLT, consisting of grade 4 thrombocytopenia. Regarding nonhematologic DLTs, 1 patient encountered grade 2 intolerable fatigue, anorexia, and dehydration, 1 had grade 3 fatigue, and 1 had grade 3 constipation (plus febrile neutropenia and grade 4 thrombocytopenia).

Among 12 patients treated on the concurrent schedule, four (33%) developed a DLT. Three patients had nonhematologic DLTs (1 grade 3 fatigue and 2 grade 3 fatigue and grade 3 dehydration), and 1 patient experienced a hematologic DLT (grade 3 febrile neutropenia).

Safety and compliance

Neutropenia and thrombocytopenia were the most frequent adverse events of at least possible attribution to study treatment (Table 2). Fatigue, nausea, diarrhea, and vomiting were the most frequent nonhematologic adverse events (Table 2). Seven patients experienced more than 1 episode



DL	No pts with DLT/Total No of pts	Nature of DLT
1	2/6	•Gr 4 thrombocytopenia, Gr 4 neutropenia > 7 days •Gr 2 intolerable fatigue, anorexia, dehydration
-1	0/3	
1a	2/3[a]	•Gr 4 thrombocytopenia •Gr 4 neutropenia > 7 days
1b	2/4	•Febrile neutropenia •Gr 3 constipation, febrile neutropenia, gr 4 thrombocytopenia
-1b	0/7	
-2b	2/7	•Gr 3 fatigue •Gr 4 thrombocytopenia
-2b	0/7	
-2c	2/3	•Gr 3 febrile neutropenia •Gr 3 fatigue
-3c	2/2	•Gr 3 fatigue, gr3 dehydration •Gr 3 fatigue, gr3 dehydration

[a]One patient did not complete cycle 1 and is not evaluable for DLT;
DL: Dose level. DLT: dose limiting toxicity

Figure 1. Dose escalation scheme for the sequential and concurrent schedules and specification of DLTs at each dose level.

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Table 2. Selected adverse events at least possibly related to study treatment by schedule and dose level, reported as percentages of patients with at least one occurrence of the event over the total number of patients treated in the respective dose level (frequencies presented refer to all treatment cycles)

	Sequential schedule						Concurrent schedule		
Adverse events and maximum grade	DL1	DL-1	DL1a	DL1b	DL-1b	DL-2b	DL-2b	DL-2c	DL-3c
	%	%	%	%	%	%	%	%	%
Neutropenia									
1/2	33	33	67	25	71	57	57	100	50
3/4	100	100	100	25	29	43	29	33	0
Thrombocytopenia									
1/2	66	100	67	25	29	71	57	33	0
3/4	33	0	33	50	0	0	0	0	0
Fatigue									
1/2	50	66	67	25	71	57	43	33	0
3/4	17	0	0	0	14	14	14	33	100
Nausea									
1/2	33	33	67	0	100	43	57	67	50
3/4	0	0	0	25	0	0	0	0	0
Vomiting									
1/2	33	0	33	25	57	29	29	67	50
3/4	0	0	0	0	0	0	0	0	0
Diarrhea									
1/2	0	33	67	0	71	43	14	0	0
3/4	0	0	0	0	0	0	0	0	0
Hyponatremia									
1/2	50	0	0	0	43	29	43	33	50
3/4	0	0	0	0	0	0	0	0	50
Dehydration									
1/2	17	0	0	0	0	0	0	0	100
3/4	17	0	0	0	0	0	0	0	100
Febrile neutropenia									
1/2	0	0	0	0	0	0	0	0	0
3/4	0	0	0	50	0	0	0	33	0
Myalgia									
1/2	0	0	0	0	14	14	0	0	0
3/4	0	0	0	0	0	0	14	0	0
Hypotension									
1/2	0	0	0	0	0	0	14	0	100
3/4	0	0	0	0	0	0	0	0	0

of grade 3 fatigue whereas other nonhematologic adverse events were mostly grade 1 or 2.

Treatment delay due to related adverse events was calculated in patients who received 2 or more cycles of treatment: on the sequential schedule, a treatment delay for 1 occasion or more occurred in 4 of 4 (100%), 2 of 2 (100%), 2 of 2 (100%), 0 of 2 (0%), 1 of 5 (20%), and 3 of 5 (60%) patients, on dose levels 1, −1, 1a, 1b, −1b, and −2b, respectively. Among patients treated on the concurrent schedule, 1 or more treatment delay occurred in 2 of 6 (33%), 1 of 1 (100%), and 1 of 1 (100%) patients on dose levels −2b, −3b, and −3c respectively.

Dose reductions or omissions occurred as follows: on the sequential schedule 4 of 6 (66%), 1 of 3 (33%), 2 of 4 (50%), 1 of 4 (25%), 2 of 7 (29%), 1 of 7 (14%) patients on dose levels 1, −1, 1a, 1b, −1b, and −2b, respectively, and on the concurrent schedule 0 of 7 (0%), 2 of 3 (66%), and 2 of 2 (100%) patients on dose levels −2b, −3b, and −3c, respectively.

Three patients treated on the sequential schedule (10%) discontinued the study due to adverse events possibly related to the treatment. These consisted of fatigue and nausea in one, nausea and neutropenia in another, and nausea and vomiting in the third. On the concurrent

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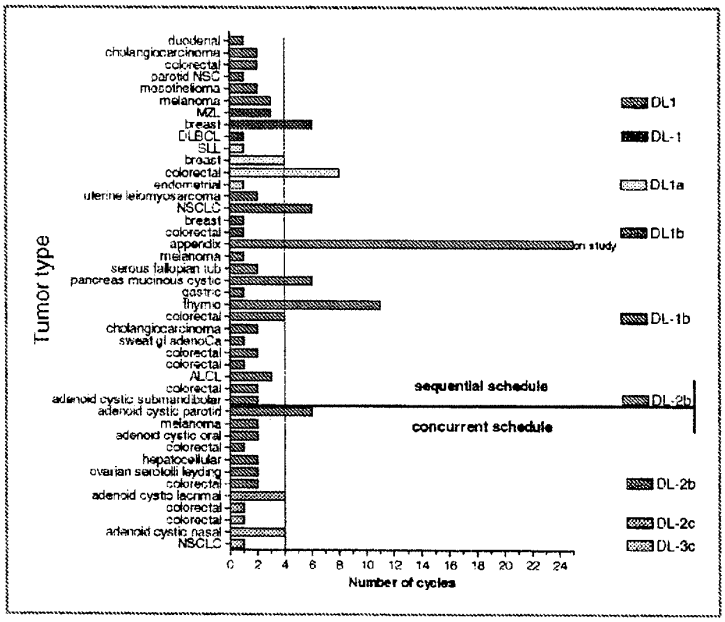

Figure 2. Number of cycles of study treatment per tumor type and dose level. DL, dose level; NSC, non small cell; MZL, marginal zone lymphoma; DLBCL, diffuse large B-cell lymphoma; SLL, small lymphocytic lymphoma; NSCLC, non-small-cell lung cancer; ALCL, anaplastic large cell lymphoma.

schedule, 2 of 12 (17%) patients discontinued treatment due to treatment related adverse events. One event was fatigue, whereas the other patient developed skin changes that were biopsied and showed a neutrophilic dermatitis consistent with Sweet syndrome, a rare syndrome that has previously been described in patients with myelodysplastic syndrome/acute leukaemia treated with the combination of HDACi and DNMTi (21). After discontinuation of treatment and a course of oral prednisone, the skin changes resolved.

Recommended phase II dose

Based on the occurrence of DLTs reported previously, the MTD for the sequential combination was decitabine 10 mg/m^2/day on days 1 to 5 with vorinostat 200 mg 3 times a day on days 6 to 12. The MTD for the concurrent schedule was decitabine 10 mg/m^2/day on days 1 to 5 with vorinostat 200 mg 2 times a day on days 3 to 9. Among the sequential schedules, the highest dose with the least treatment delay was achieved in dose level −1b (decitabine 10 mg/m^2/day on days 1–5 with vorinostat 200 mg twice daily, days 6–12). This is also the dose level with the highest percentage of patients with stable disease for 4 cycles or more. In fact, 4 of the 11 patients (36%) who remained on study for 4 or more cycles were treated at this dose level. Thus, this dose level represents the recommended phase II dose (RP2D) for the combination of the two drugs.

Antitumor activity

No objective tumor responses were observed. Among 38 patients evaluable for response, 11 (29%) with previously progressive cancers had stable disease for 4 or more cycles of treatment. Three of 11 had stable disease for 8 or more cycles, including 1 patient with colon cancer (8 cycles), 1 patient with thymoma (11 cycles), and 1 patient with mucinous adenocarcinoma of the appendix who remains on study after 25 cycles (Fig. 2).

PK analyses

PK results (Tables 3 and 4) of decitabine were similar to those reported in previous studies (22). Decitabine is rapidly eliminated with a half-life of approximately 20 minutes. On the sequential schedule, increasing doses of decitabine did not produce dose proportional increases in maximum plasma concentration (C_{max}) and area under the plasma concentration–time curve (AUC). No significant differences were found when comparing PK parameters on day 1 and day 5 within the same dose level in either schedule. In addition, there were no statistically significant differences when comparing PK parameters for

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Table 3. Decitabine PK parameters

	Sequential schedule					
Dose (no.of pts)	**10 mg/m² (12)**		**15 mg/m² (3)**		**20 mg/m² (4)**	
PK parameter	**Day 1**	**Day 5**	**Day 1**	**Day 5**	**Day 1**	**Day 5**
C_{max} (ng/mL)	57.5 (27.5)	67.5 (24.5)	129.03 (74.25)	142.33 (26.16)	134.88 (31.52)	198.00 (42.86)
AUC_t (ng.h/mL)	47.2 (22.0)	55.8 (21.0)	112.11 (67.59)	113.99 (18.45)	115.38 (18.58)	160.67 (40.40)
AUC_{inf} (ng.h/mL)	48.4 (22.1)	57.8 (21.4)	115.68 (69.62)	116.33 (19.10)	119.30 (18.24)	164.55 (41.52)
$t_{1/2}$ (h)	0.34 (0.11)	0.36 (0.14)	0.38 (0.05)	0.33 (0.03)	0.36 (0.06)	0.42 (0.11)
CL (l/h)	456 (217)	368 (170)	340 (270)	236 (37)	317 (81)	235 (73)
	Concurrent schedule					
Dose [no. of pts]	**10 mg/m² [12]**		-		-	
PK parameter	Day 1	Day 5				
C_{max} (ng/mL)	88.63 (43.34)	62.91 (18.36)				
AUC_t (ng.h/mL)	75.78 (49.59)	56.27 (11.68)				
AUC_{inf} (ng.h/mL)	77.57 (51.12)	57.44 (11.68)				
$t_{1/2}$ (h)	0.27 (0.06)	0.27 (0.07)				
CL(l/h)	280 (79)	334 (80)				

NOTE: Values reported: Mean (SD)
Abbreviations: $t_{1/2}$, terminal half-life; AUC_t, area under the plasma decitabine concentration versus time curve to the last sampling time; AUC_{inf}, area under the plasma decitabine concentration versus time curve from zero to infinity; CL, clearance.

the dose of 10 mg/m²/day between the 2 schedules of administration.

A comparison of vorinostat PK parameters with increasing doses was possible only for the sequential schedule. AUC and C_{max} after a single vorinostat administration increased proportionaly when the dose of vorinostat was increased from 100 mg to 200 mg. Interestingly, C_{max}, AUC_{inf} (= AUC to infinity), and AUC_t (= AUC over

Table 4. Vorinostat PK parameters

	Sequential schedule		
Dose (no. of pts)	**100 mg BID (13)**	**200 mg BID (10)**	**200 mg TID (7)**
PK parameter			
C_{max} (ng/mL)	165 (88)	313 (130)	293 (150)
AUC_t (ng.h/mL)	424 (163)	1,021 (251)	1,086 (619)
AUC_{inf} (ng.h/mL)	446 (158)	1,184 (276)	1,352 (873)
$t_{1/2}$ (h)	1.39 (0.48)	2.55 (1.99)	1.71 (0.94)
CL/F (ml/min)	4.3 (2.3)	3 (0.7)	4 (3.7)
		Concurrent schedule	
dose (no. of pts)	-	200 mg BID (10)	200 mg TID (2)
PK parameter			
C_{max} (ng/mL)		214 (114)	227 (378)
AUC_t (ng.h/mL)		573 (216)	683 (1,518)
AUC_{inf} (ng.h/mL)		708 (235)	NA
$t_{1/2}$ (h)		2.39 (1.45)	NA
CL/F (mL/min)		5.3 (2.3)	NA

NOTE: Values reported: Mean (SD)
Abbreviations: $t_{1/2}$, terminal half-life; AUCt, area under the plasma vorinostat concentration versus time curve to the last sampling time; AUC_{inf}, area under the plasma vorinostat concentration versus time curve from zero to infinity; CL, clearance.

Published OnlineFirst January 28, 2011; DOI:10.1158/1078-0432.CCR-10-1893

dosing interval) were lower in the concurrent schedule than those in the sequential schedule ($P = 0.09$, 0.004, and 0.0005, respectively) at the 200 mg BID dose level. The difference in the AUC_1 remains statistically significant after adjustment for multiple comparisons (Supplementary Figure A1).

Discussion

Aberrant DNA methylation and histone deacetylation are involved in tumor formation and progression and have been evaluated as targets for the development of anticancer agents (1, 2). The possibility of optimally reexpressing methylated genes following treatment with the combination of a DNMTi with an HDACi has been confirmed in preclinical studies and formed the basis for clinical trials using combined epigenetic therapies (23).

Here we report for the first time the results of a phase I trial demonstrating the feasibility of delivering decitabine in combination with vorinostat in patients with advanced solid tumors or NHLs. Decitabine given for 5 days at a dose of 10 mg/m^2/day as a 1-hour intravenous infusion can be combined with oral vorinostat either on a sequential (vorinostat 200 mg 3 times a day on days 6–12) or a concurrent schedule (vorinostat 200 mg 2 times a day on days 3–9). The toxicities observed were predictable and manageable at these stated MTD doses. In both schedules, the combination of decitabine and vorinostat appears to have a narrow therapeutic index and both drugs required dose reductions from their single-agent recommended doses used in previous studies of hematologic malignancies and solid tumors. However, the optimal single-agent doses of decitabine and vorinostat in patients with solid tumors remain unknown and there is no clear evidence that higher doses are associated with better outcome.

Among the 2 schedules evaluated in this study, the sequential schedule appears to be easier to deliver and more tolerable. In addition, PK analyses showed that for the same dose of vorinostat, AUC, and C_{max} were lower for the concurrent schedule, suggesting a possible unfavorable PK interaction between the 2 drugs. However, the number of patients enrolled in our study was small and the study was not designed to establish if this was due to increased metabolism or reduced absorption of vorinostat. Notably, escalation of vorinostat to 200 mg thrice a day on the concurrent schedule resulted in increased toxicities with 2 of 2 patients developing DLTs. Within the sequential schedule, dose level 1b (decitabine 10 mg/m^2/day on days 1–5 and vorinostat 200 mg twice a day on days 6–12) was more favorable in dose delivery without delays, and therefore it represents the dose we recommend for further phase II evaluation. Dose level −1b on the sequential schedule also had the highest percentage of patients achieving stable disease for 4 cycles or more. Although this represents only a small number of patients, rendering it impossible to draw any definitive conclusion regarding superiority of any of the dose levels studied, we believe this dose level warrants further investigation in clinical trials.

Combination therapies employing DNMTi or HDACi with other agents are being pursued clinically (8, 9). A small number of clinical trials have evaluated different combinations of epigenetic agents in patients with hematologic and, more recently, solid malignancies (22, 24–27). There is optimism that combined epigenetic therapy can result in increased antitumor activity in comparison to the use of single-agent DNMTi or HDACi, but this needs validation in randomized studies. In our study we observed stable disease in previously progressing patients with different tumor types, but it is not possible to establish if this is due to the combination of the 2 agents and what the expected outcomes would be if each agent was used alone. Decitabine and vorinostat are active in hematologic malignancies and cutaneous T-cell lymphomas, respectively, but their role in the treatment of solid tumors remains undefined. Among the 2 agents, vorinostat has shown single-agent antitumor activity with reports of stable disease and a few cases of partial responses in patients with different types of solid tumors (16, 28).

This was a small phase I study and it is difficult to speculate based on its results in which tumor types this combination should be further explored. Recently reported studies of vorinostat in patients with solid tumors as single-agent or in combination with chemotherapy have shown preliminary evidence of activity in non–small-cell lung, breast, colorectal, mesothelioma, thyroid, and adenoid cystic carcinoma (16, 28–30). It is therefore reasonable to consider further clinical investigation of this combination in the context of the aforementioned malignancies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interests were disclosed.

Grant Support

Supported by NCI Grants, U01CA132123, U01CA099168, and P30-CA47904

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Received July 17, 2010; revised October 8, 2010; accepted October 8, 2010; published OnlineFirst January 28, 2011.

References

1. Esteller M. Epigenetics in cancer. N Engl J Med 2008;358:1148–59.
2. Jones PA, Baylin SB. The epigenomics of cancer. Cell 2007;128:683–92.
3. Plekarz RL, Bates SE. Epigenetic modifiers: basic understanding and clinical development. Clin Cancer Res 2009;15:3918–26.
4. Wilson VL, Jones PA, Momparler RL. Inhibition of DNA methylation in L1210 leukemic cells by 5-aza-2'-deoxycytidine as a possible mechanism of chemotherapeutic action. Cancer Res 1983;43:3493–6.

Published OnlineFirst January 28, 2011; DOI:10.1158/1078-0432.CCR-10-1893

5. Finnin MS, Donigian JR, Cohen A, Richon VM, Rifkind RA, Marks PA, et al. Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors. Nature 1999;401:188–93.
6. Kantarjian H, Issa JP, Rosenfeld CS, Bennett JM, Albitar M, DiPersio J, et al. Decitabine improves patient outcomes in myelodysplastic syndromes: results of a phase III randomized study. Cancer 2006;106:1794–603.
7. Mann BS, Johnson JR, Cohen MH, Justice R, Pazdur R. FDA approval summary: vorinostat for treatment of advanced primary cutaneous T-cell lymphoma. Oncologist 2007;12:1247–52.
8. Lane AA, Chabner BA. Histone deacetylase inhibitors in cancer therapy. J Clin Oncol 2009;27:5459–68.
9. Issa JP, Kantarjian HM. Targeting DNA methylation. Clin Cancer Res 2009;15:3938–46.
10. Cameron EE, Bachman KE, Myohanen S, Herman JG, Baylin SB. Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. Nat Genet 1999;21:103–7.
11. Kikuchi T, Toyota M, Itoh F, Suzuki H, Obata T, Yamamoto H, et al. Inactivation of p57KIP2 by regional promoter hypermethylation and histone deacetylation in human tumors. Oncogene 2002;21:2741–9.
12. Boivin AJ, Momparler LF, Hurtubise A, Momparler RL. Antineoplastic action of 5-aza-2′-deoxycytidine and phenylbutyrate on human lung carcinoma cells. Anticancer Drugs 2002;13:869–74.
13. Zhu WG, Lakshmanan RR, Beal MD, Otterson GA. DNA methyltransferase inhibition enhances apoptosis induced by histone deacetylase inhibitors. Cancer Res 2001;61:1327–33.
14. Yang H, Hoshino K, Sanchez-Gonzalez B, Kantarjian H, Garcia-Manero G. Antileukemia activity of the combination of 5-aza-2′-deoxycytidine with valproic acid. Leuk Res 2005;29:739–48.
15. Kantarjian HM, O'Brien S, Shan J, Aribi A, Garcia-Manero G, Jabbour E, et al. Update of the decitabine experience in higher risk myelodysplastic syndrome and analysis of prognostic factors associated with outcome. Cancer 2007;109:265–73.
16. Kelly WK, O'Connor OA, Krug LM, Chiao JH, Heaney M, Curley T, et al. Phase I study of an oral histone deacetylase inhibitor, suberoylanilide hydroxamic acid, in patients with advanced cancer. J Clin Oncol 2005;23:3923–31.
17. Garcia-Manero G, Yang H, Bueso-Ramos C, Ferrajoli A, Cortes J, Wierda WG, et al. Phase 1 study of the histone deacetylase inhibitor vorinostat (suberoylanilide hydroxamic acid [SAHA]) in patients with advanced leukemias and myelodysplastic syndromes. Blood 2008;111:1060–6.
18. Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, et al. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst 2000;92:205–16.
19. Liu Z, Marcucci G, Byrd JC, Grever M, Xiao J, Chan KK. Characterization of decomposition products and preclinical and low dose clinical pharmacokinetics of decitabine (5-aza-2′-deoxycytidine) by a new liquid chromatography/tandem mass spectrometry quantification method. Rapid Commun Mass Spectrom 2006;20:1117–26.
20. Parise RA, Holleran JL, Beumer JH, Ramalingam S, Egorin MJ. A liquid chromatography-electrospray ionization tandem mass spectrometric assay for quantitation of the histone deacetylase inhibitor, vorinostat (suberoylanilide hydroxamic acid, SAHA), and its metabolites in human serum. J Chromatogr B Analyt Technol Biomed Life Sci 2006;840:108–15.
21. Alencar C, Abramowtiz M, Parekh S, Braunshweig I, Jacobson M, Silverman L, et al. Atypical presentations of Sweet's syndrome in patients with MDS/AML receiving combinations of hypomethylating agents with histone deacetylase inhibitors. Am J Hematol 2009;84:688–9.
22. Blum W, Klisovic RB, Hackanson B, Liu Z, Liu S, Devine H, et al. Phase I study of decitabine alone or in combination with valproic acid in acute myeloid leukemia. J Clin Oncol 2007;25:3884–91.
23. Bots M, Johnstone RW. Rational combinations using HDAC inhibitors. Clin Cancer Res 2009;15:3970–7.
24. Garcia-Manero G, Kantarjian HM, Sanchez-Gonzalez B, Yang H, Rosner G, Verstovsek S, et al. Phase 1/2 study of the combination of 5-aza-2′-deoxycytidine with valproic acid in patients with leukemia. Blood 2006;108:3271–9.
25. Gore SD, Baylin S, Sugar E, Carraway H, Miller CB, Carducci M, et al. Combined DNA methyltransferase and histone deacetylase inhibition in the treatment of myeloid neoplasms. Cancer Res 2006;66:6361–9.
26. Braitch F, Soriano AO, Garcia-Manero G, Hong D, Johnson MM, Silva Lde P, et al. Phase I study of epigenetic modulation with 5-azacytidine and valproic acid in patients with advanced cancers. Clin Cancer Res 2008;14:6296–301.
27. Lin J, Gilbert J, Rudek MA, Zwiebel JA, Gore S, Jiemjit A, et al. A phase I dose-finding study of 5-azacytidine in combination with sodium phenylbutyrate in patients with refractory solid tumors. Clin Cancer Res 2009;15:6241–9.
28. Luu TH, Morgan RJ, Leong L, Lim D, McNamara M, Portnow J, et al. A phase II trial of vorinostat (suberoylanilide hydroxamic acid) in metastatic breast cancer: a California Cancer Consortium study. Clin Cancer Res 2008;14:7138–42.
29. Ramalingam SS, Maitland ML, Frankel P, Argiris AE, Koczywas M, Gitlitz B, et al. Carboplatin and Paclitaxel in combination with either vorinostat or placebo for first-line therapy of advanced non-small-cell lung cancer. J Clin Oncol 2010;28:56–62.
30. Fakih MG, Fetterly G, Egorin MJ, Mulndi JR, Espinoza-Delgado I, Zwiebel JA, et al. A phase I, pharmacokinetic, and pharmacodynamic study of two schedules of vorinostat in combination with 5-fluorouracil and leucovorin in patients with refractory solid tumors. Clin Cancer Res 2010;16:3786–94.

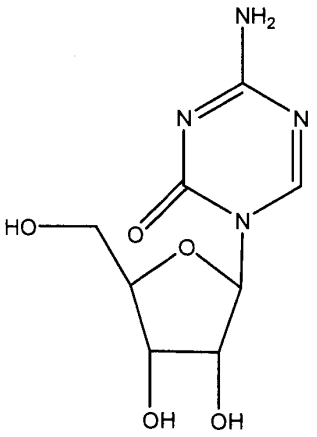
Version: 5-18-04

Vidaza™
(azacitidine for injectable suspension) R_x only

For subcutaneous use only

DESCRIPTION

Vidaza™ (azacitidine for injectable suspension) contains azacitidine, which is a pyrimidine nucleoside analog of cytidine. Azacitidine is 4-amino-1-β-D-ribofuranosyl-s-triazin-2(1*H*)-one. The structural formula is as follows:



The empirical formula is $C_8H_{12}N_4O_5$. The molecular weight is 244. Azacitidine is a white to off-white solid. Azacitidine was found to be insoluble in acetone, ethanol, and methyl ethyl ketone; slightly soluble in ethanol/water (50/50), propylene glycol, and polyethylene glycol; sparingly soluble in water, water saturated octanol, 5% dextrose in water, N-methyl-2-pyrrolidone, normal saline and 5% Tween 80 in water; and soluble in dimethylsulfoxide (DMSO).

The finished product is supplied in a sterile form for reconstitution and subcutaneous injection only. Vials of Vidaza contain 100 mg of azacitidine and 100 mg mannitol as a sterile lyophilized powder.

CLINICAL PHARMACOLOGY

Mechanism of Action

Vidaza is believed to exert its antineoplastic effects by causing hypomethylation of DNA and direct cytotoxicity on abnormal hematopoietic cells in the bone marrow. The concentration of azacitidine required for maximum inhibition of DNA methylation *in vitro* does not cause major suppression of DNA synthesis. Hypomethylation may restore normal function to genes that are

critical for differentiation and proliferation. The cytotoxic effects of azacitidine cause the death of rapidly dividing cells, including cancer cells that are no longer responsive to normal growth control mechanisms. Non-proliferating cells are relatively insensitive to Vidaza.

Pharmacokinetics

The pharmacokinetics of azacitidine were studied in six MDS patients following a single 75 mg/m^2 subcutaneous (SC) dose and a single 75 mg/m^2 intravenous (IV) dose. Azacitidine is rapidly absorbed after SC administration; the peak plasma azacitidine concentration of 750 ± 403 ng/ml occurred in 0.5 hour. The bioavailability of SC azacitidine relative to IV azacitidine is approximately 89%, based on area under the curve. Mean volume of distribution following IV dosing is 76 ± 26 L. Mean apparent SC clearance is 167 ± 49 L/hour and mean half-life after SC administration is 41 ± 8 minutes.

Published studies indicate that urinary excretion is the primary route of elimination of azacitidine and its metabolites. Following IV administration of radioactive azacitidine to 5 cancer patients, the cumulative urinary excretion was 85% of the radioactive dose. Fecal excretion accounted for <1% of administered radioactivity over three days. Mean excretion of radioactivity in urine following SC administration of ^{14}C-azacitidine was 50%. The mean elimination half-lives of total radioactivity (azacitidine and its metabolites) were similar after IV and SC administrations, about 4 hours.

Special Populations

The effects of renal or hepatic impairment, gender, age, or race on the pharmacokinetics of azacitidine have not been studied (see **CONTRAINDICATIONS, PRECAUTIONS** and **DOSAGE AND ADMINISTRATION**).

Drug-Drug Interactions

Drug interaction studies with azacitidine have not been conducted.

An *in vitro* study of azacitidine incubation in human liver fractions indicated that azacitidine may be metabolized by the liver. Whether azacitidine metabolism may be affected by known microsomal enzyme inhibitors or inducers has not been studied.

The potential of azacitidine to inhibit cytochrome P450 (CYP) enzymes is not known.

In vitro studies with human cultured hepatocytes indicate that azacitidine at concentrations of 1.0 μM to 100 μM does not induce CYP 1A2, 2C19, or 3A4/5.

CLINICAL STUDIES

A randomized, open-label, controlled trial carried out in 51 U.S. and 2 Canadian sites compared the safety and efficacy of subcutaneous Vidaza plus supportive care with supportive care alone ("observation") in patients with any of the five FAB subtypes of myelodysplastic syndromes

(MDS): refractory anemia (RA), RA with ringed sideroblasts (RARS), RA with excess blasts (RAEB), RAEB in transformation (RAEB-T), and chronic myelomonocytic leukemia (CMMoL). RA and RARS patients were included if they met one or more of the following criteria: required packed RBC transfusions; had platelet counts $\leq 50.0 \times 10^9$/L; required platelet transfusions; or were neutropenic (ANC $< 1.0 \times 10^9$/L) with infections requiring treatment with antibiotics. Patients with acute myelogenous leukemia (AML) were not intended to be included. Baseline patient and disease characteristics are summarized in Table 1; the 2 groups were similar.

Vidaza was administered at a subcutaneous dose of 75 mg/m^2 daily for seven days every four weeks. The dose was increased to 100 mg/m^2 if no beneficial effect was seen after two treatment cycles. The dose was decreased and/or delayed based on hematologic response or evidence of renal toxicity. Patients in the observation arm were allowed by protocol to cross over to Vidaza if they had increases in bone marrow blasts, decreases in hemoglobin, increases in red cell transfusion requirements, or decreases in platelets, or if they required a platelet transfusion or developed a clinical infection requiring treatment with antibiotics. For purposes of assessing efficacy, the primary endpoint was response rate (as defined in Table 2).

Of the 191 patients included in the study, independent review (adjudicated diagnosis) found that 19 had the diagnosis of AML at baseline. These patients were excluded from the primary analysis of response rate, although they were included in an intent-to-treat (ITT) analysis of all patients randomized. Approximately 55% of the patients randomized to observation crossed over to receive Vidaza treatment.

Table 1. Baseline Demographics and Disease Characteristics

	Vidaza (N=99)	Observation (N=92)
Gender (n%)		
Male	72 (72.7)	60 (65.2)
Female	27 (27.3)	32 (34.8)
Race (n%)		
White	93 (93.9)	85 (92.4)
Black	1 (1.0)	1 (1.1)
Hispanic	3 (3.0)	5 (5.4)
Asian/Oriental	2 (2.0)	1 (1.1)
Age (years)		
N	99	91
Mean ± SD	67.3 ± 10.39	68.0 ± 10.23
Range	31 - 92	35 - 88
Adjudicated MDS diagnosis at study entry (n%)		
RA	21 (21.2)	18 (19.6)
RARS	6 (6.1)	5 (5.4)
RAEB	38 (38.4)	39 (42.4)
RAEB-T	16 (16.2)	14 (15.2)
CMMoL	8 (8.1)	7 (7.6)
AML	10 (10.1)	9 (9.8)
Transfusion product used in 3 months before study entry (n%)		
Any transfusion product	70 (70.7)	59 (64.1)
Blood cells, packed human	66 (66.7)	55 (59.8)
Platelets, human blood	15 (15.2)	12 (13.0)
Hetastarch	0(0.0)	1(1.1)
Plasma protein fraction	1(1.0)	0(0.0)
Other	2(2.0)	2(2.2)

Table 2. Response Criteria

		RA	RARS	RAEB	RAEB-T	CMMoL
Complete Response (CR), duration ≥ 4 weeks	**Marrow**	< 5% blasts				
	Peripheral Blood	Normal CBC if abnormal at baseline Absence of blasts in the peripheral circulation				
Partial Response (PR), duration ≥ 4 weeks	**Marrow**	No marrow requirements		≥ 50% decrease in blasts Improvement of marrow dyspoiesis		
	Peripheral Blood	≥ 50% restoration in the deficit from normal levels of baseline white cells, hemoglobin and platelets if abnormal at baseline No blasts in the peripheral circulation For CMMoL, if WBC is elevated at baseline, a ≥ 75% reduction in the excess count over the upper limit of normal				

The overall response rate (CR +PR) of 15.7% in Vidaza-treated patients without AML (16.2% for all Vidaza randomized patients including AML) was statistically significantly higher than the response rate of 0% in the observation group (p=<0.0001) (Table 3). The majority of patients who achieved either CR or PR had either 2 or 3 cell line abnormalities at baseline (79%; 11/14) and had elevated bone marrow blasts or were transfusion dependent at baseline. Patients responding to Vidaza had a decrease in bone marrow blasts percentage, or an increase in platelets, hemoglobin or WBC. Greater than 90% of the responders initially demonstrated these changes by the 5th treatment cycle. All patients who had been transfusion dependent became transfusion independent during PR or CR. The mean and median duration of clinical response of PR or better was estimated as 512 and 330 days, respectively; 75% of the responding patients were still in PR or better at completion of treatment. Response occurred in all MDS subtypes as well as in patients with adjudicated baseline diagnosis of AML.

Table 3. Response Rates

	Vidaza (N=89)	Observation Before Crossover (N=83)	
Response	**n (%)**	**N (%)**	**P value**
Overall (CR+PR)	14 (15.7)	0 (0.0)	(<0.0001)
Complete (CR)	5 (5.6)	0 (0.0)	(0.06)
Partial (PR)	9 (10.1)	0 (0.0)	--

Patients in the observation group who crossed over to receive Vidaza treatment (47 patients) had a response rate of 12.8%.

A multi-center, open-label, single-arm study of 72 patients with RAEB, RAEB-T, CMMoL, or AML was also carried out. Treatment with subcutaneous Vidaza resulted in a response rate (CR + PR) of 13.9%, using criteria similar to those described above. The mean and median duration of clinical response of PR or better was estimated as 810 and 430 days, respectively; 80% of the responding patients were still in PR or better at the time of completion of study involvement. In another open-label, single-arm study of 48 patients with RAEB, RAEB-T, or AML, treatment with intravenous Vidaza resulted in a response rate of 18.8%, again using criteria similar to those described above. The mean and median duration of clinical response of PR or better was estimated as 389 and 281 days, respectively; 67% of the responding patients were still in PR or better at the time of completion of treatment. Response occurred in all MDS subtypes as well as in patients with adjudicated baseline diagnosis of AML in both of these studies. Vidaza dosage regimens in these 2 studies were similar to the regimen used in the controlled study.

Benefit was seen in patients who did not meet the criteria for PR or better, but were considered "improved." About 24% of Vidaza-treated patients were considered improved, and about 2/3 of those lost transfusion dependence. In the observation group, only 5/83 patients met criteria for improvement; none lost transfusion dependence. In all three studies, about 19% of patients met criteria for improvement with a median duration of 195 days.

Response rate estimates were similar regardless of age or gender.

INDICATIONS AND USAGE

Vidaza is indicated for treatment of patients with the following myelodysplastic syndrome subtypes: refractory anemia or refractory anemia with ringed sideroblasts (if accompanied by neutropenia or thrombocytopenia or requiring transfusions), refractory anemia with excess blasts, refractory anemia with excess blasts in transformation, and chronic myelomonocytic leukemia.

CONTRAINDICATIONS

Vidaza is contraindicated in patients with a known hypersensitivity to azacitidine or mannitol. Vidaza is also contraindicated in patients with advanced malignant hepatic tumors. (See **PRECAUTIONS**).

WARNINGS

Pregnancy - Teratogenic Effects: Pregnancy Category D

Vidaza may cause fetal harm when administered to a pregnant woman. Early embryotoxicity studies in mice revealed a 44% frequency of intrauterine embryonal death (increased resorption) after a single IP (intraperitoneal) injection of 6 mg/m^2 (approximately 8% of the recommended human daily dose on a mg/m^2 basis) azacitidine on gestation day 10. Developmental abnormalities in the brain have been detected in mice given azacitidine on or before gestation day 15 at doses of ~3-12 mg/m^2 (approximately 4%-16% the recommended human daily dose on a mg/m^2 basis).

In rats, azacitidine was clearly embryotoxic when given IP on gestation days 4-8 (postimplantation) at a dose of 6 mg/m^2 (approximately 8% of the recommended human daily dose on a mg/m^2 basis), although treatment in the preimplantation period (on gestation days 1-3) had no adverse effect on the embryos. Azacitidine caused multiple fetal abnormalities in rats after a single IP dose of 3 to 12 mg/m^2 (approximately 8% the recommended human daily dose on a mg/m^2 basis) given on gestation day 9, 10, 11 or 12. In this study azacitidine caused fetal death when administered at 3-12 mg/m^2 on gestation days 9 and 10; average live animals per litter was reduced to 9% of control at the highest dose on gestation day 9. Fetal anomalies included: CNS anomalies (exencephaly/encephalocele), limb anomalies (micromelia, club foot, syndactyly, oligodactyly), and others (micrognathia, gastroschisis, edema, and rib abnormalities).

There are no adequate and well-controlled studies in pregnant women using Vidaza. If this drug is used during pregnancy, or if the patient becomes pregnant while taking this drug, the patient should be apprised of the potential hazard to the fetus.

Women of childbearing potential should be advised to avoid becoming pregnant while receiving treatment with Vidaza.

Use in Males

Men should be advised to not father a child while receiving treatment with Vidaza. (See **PRECAUTIONS: Carcinogenesis, Mutagenesis, Impairment of Fertility** for discussion of pre-mating effects of azacitidine exposure on male fertility and embryonic viability.)

PRECAUTIONS

General

Treatment with Vidaza is associated with neutropenia and thrombocytopenia. Complete blood counts should be performed as needed to monitor response and toxicity, but at a minimum, prior to each dosing cycle. After administration of the recommended dosage for the first cycle, dosage for subsequent cycles should be reduced or delayed based on nadir counts and hematologic response as described in **DOSAGE AND ADMINISTRATION**.

Safety and effectiveness of Vidaza in patients with MDS and hepatic or renal impairment have not been studied as these patients were excluded from the clinical trials.

Because azacitidine is potentially hepatotoxic in patients with severe pre-existing hepatic impairment, caution is needed in patients with liver disease. Patients with extensive tumor burden due to metastatic disease have been rarely reported to experience progressive hepatic coma and death during azacitidine treatment, especially in such patients with baseline albumin <30 g/L. Azacitidine is contraindicated in patients with advanced malignant hepatic tumors (See **CONTRAINDICATIONS**).

Renal abnormalities ranging from elevated serum creatinine to renal failure and death have been reported rarely in patients treated with intravenous azacitidine in combination with other chemotherapeutic agents for non-MDS conditions. In addition, renal tubular acidosis, defined as a fall in serum bicarbonate to <20 mEq/L in association with an alkaline urine and hypokalemia (serum potassium <3 mEq/L) developed in 5 patients with CML treated with azacitidine and etoposide. If unexplained reductions in serum bicarbonate <20 mEq/L or elevations of BUN or serum creatinine occur, the dosage should be reduced or held as described in **DOSAGE AND ADMINISTRATION.**

Patients with renal impairment should be closely monitored for toxicity since azacitidine and its metabolites are primarily excreted by the kidneys (see **DOSAGE AND ADMINISTRATION** section).

Information for Patients

Patients should inform their physician about any underlying liver or renal disease.

Women of childbearing potential should be advised to avoid becoming pregnant while receiving treatment with Vidaza.

Men should be advised to not father a child while receiving treatment with Vidaza.

Laboratory Tests

Complete blood counts should be performed as needed to monitor response and toxicity, but at a minimum, prior to each cycle. Liver chemistries and serum creatinine should be obtained prior to initiation of therapy.

Drug Interactions

No formal assessments of drug-drug interactions between Vidaza and other agents have been conducted. (See **CLINICAL PHARMACOLOGY**.)

Carcinogenesis, Mutagenesis, Impairment of Fertility

The potential carcinogenicity of azacitidine was evaluated in mice and rats. Azacitidine induced tumors of the hematopoietic system in female mice at 2.2 mg/kg (6.6 mg/m^2, approximately 8% the recommended human daily dose on a mg/m^2 basis) administered IP three times per week for 52 weeks. An increased incidence of tumors in the lymphoreticular system, lung, mammary gland, and skin was seen in mice treated with azacitidine IP at 2.0 mg/kg (6.0 mg/m^2, approximately 8% the recommended human daily dose on a mg/m^2 basis) once a week for 50 weeks. A tumorigenicity study in rats dosed twice weekly at 15 or 60 mg/m^2 (approximately 20-80% the recommended human daily dose on a mg/m^2 basis) revealed an increased incidence of testicular tumors compared with controls.

The mutagenic and clastogenic potential of azacitidine was tested in *in vitro* bacterial systems *Salmonella typhimurium* strains TA100 and several strains of *trpE8*, *Escherichia coli* strains WP14 Pro, WP3103P, WP3104P, and CC103; in *in vitro* forward gene mutation assay in mouse lymphoma cells and human lymphoblast cells; and in an *in vitro* micronucleus assay in mouse L5178Y lymphoma cells and Syrian hamster embryo cells. Azacitidine was mutagenic in bacterial and mammalian cell systems. The clastogenic effect of azacitidine was shown by the induction of micronuclei in L5178Y mouse cells and Syrian hamster embryo cells.

Administration of azacitidine to male mice at 9.9 mg/m^2 (approximately 9% the recommended human daily dose on a mg/m^2 basis) daily for 3 days prior to mating with untreated female mice resulted in decreased fertility and loss of offspring during subsequent embryonic and postnatal development. Treatment of male rats three times per week for 11 or 16 weeks at doses of 15 to 30 mg/m^2 (approximately 20-40%, the recommended human daily dose on a mg/m^2 basis) resulted in decreased weight of the testes and epididymides, and decreased sperm counts accompanied by decreased pregnancy rates and increased loss of embryos in mated females. In a related study, male rats treated for 16 weeks at 24 mg/m^2 resulted in an increase in abnormal embryos in mated females when examined on day 2 of gestation. See **WARNINGS**.

Pregnancy

Teratogenic Effects: Pregnancy Category D. See **WARNINGS** section.

Nursing Mothers

It is not known whether azacitidine or its metabolites are excreted in human milk. Because of the potential for tumorigenicity shown for azacitidine in animal studies and the potential for serious adverse reactions, women treated with azacitidine should not nurse.

Pediatric Use

Safety and effectiveness in pediatric patients have not been established.

Geriatric Use

Of the total number of patients in the three clinical studies described in **CLINICAL STUDIES**, above, 62 percent were 65 years and older and 21 percent were 75 years and older. No overall differences in effectiveness were observed between these patients and younger patients. In addition there were no relevant differences in the frequency of adverse events observed in patients 65 years and older compared to younger patients.

Azacitidine and its metabolites are known to be substantially excreted by the kidney, and the risk of toxic reactions to this drug may be greater in patients with impaired renal function. Because elderly patients are more likely to have decreased renal function, it may be useful to monitor renal function (see **DOSAGE AND ADMINISTRATION** section).

ADVERSE REACTIONS

Overview

Adverse Reactions Described in Other Labeling Sections: neutropenia, thrombocytopenia, elevated serum creatinine, renal failure, renal tubular acidosis, hypokalemia, hepatic coma.

Most Commonly Occurring Adverse Reactions (SC Route): nausea, anemia, thrombocytopenia, vomiting, pyrexia, leukopenia, diarrhea, fatigue, injection site erythema, constipation, neutropenia, ecchymosis.

Adverse Reactions Most Frequently (>2%) Resulting in Clinical Intervention (SC Route):
Discontinuation: leukopenia (5.0%), thrombocytopenia (3.6%), neutropenia (2.7%).
Dose Held: leukopenia (4.5%), neutropenia (4.5%), febrile neutropenia (2.7%).
Dose Reduced: leukopenia (4.5%), neutropenia (4.1%), thrombocytopenia (3.2%).

Discussion of Adverse Reactions Information

The data described below reflect exposure to Vidaza in 268 patients, including 116 exposed for 6 cycles (approximately 6 months) or more and 60 exposed for greater than 12 cycles (approximately one year). Vidaza was studied primarily in supportive care-controlled and uncontrolled trials (n= 150 and n=118, respectively). The population in the subcutaneous studies (n = 220) was 23 to 92 years old (mean 66.4 years), 68% male, and 94% white, and had MDS or AML. The population in the IV study (n = 48) was 35 to 81 years old (mean 63.1 years), 65% male, and 100% white. Most patients received average daily doses between 50 and 100 mg/m^2.

The following table presents the most common adverse events, whether or not considered drug related by investigators, occurring in at least 5% of patients treated with Vidaza in the supportive care-controlled trial and the uncontrolled subcutaneous trial combined. It is important to note that duration of exposure was longer for the Vidaza-treated group than for the observation group: patients received Vidaza for a mean of 11.4 months while mean time in the observation arm was 6.1 months.

Table 4: Most Frequently Observed Adverse Events (≥ 5% in All Vidaza)*

Preferred Term**	All Vidaza‡ (N=220)	Observation† (N=92)
At least 1 TEAE	219 (99.5)	89 (96.7)
Nausea	155 (70.5)	16 (17.4)
Anemia	153 (69.5)	59 (64.1)
Thrombocytopenia	144 (65.5)	42 (45.7)
Vomiting	119 (54.1)	5 (5.4)
Pyrexia	114 (51.8)	28 (30.4)
Leukopenia	106 (48.2)	27 (29.3)
Diarrhea	80 (36.4)	13 (14.1)
Fatigue	79 (35.9)	23 (25.0)
Injection site erythema	77 (35.0)	0
Constipation	74 (33.6)	6 (6.5)
Neutropenia	71 (32.3)	10 (10.9)
Ecchymosis	67 (30.5)	14 (15.2)
Cough	65 (29.5)	14 (15.2)
Dyspnea	64 (29.1)	11 (12.0)
Weakness	64 (29.1)	19 (20.7)
Rigors	56 (25.5)	10 (10.9)
Petechiae	52 (23.6)	8 (8.7)
Injection site pain	50 (22.7)	0
Arthralgia	49 (22.3)	3 (3.3)
Headache	48 (21.8)	10 (10.9)
Anorexia	45 (20.5)	6 (6.5)
Pain in limb	44 (20.0)	5 (5.4)
Pharyngitis	44 (20.0)	7 (7.6)
Back pain	41 (18.6)	7 (7.6)
Contusion	41 (18.6)	9 (9.8)
Dizziness	41 (18.6)	5 (5.4)

Preferred Term**	All Vidaza‡ (N=220)	Observation† (N=92)
At least 1 TEAE	219 (99.5)	89 (96.7)
Edema peripheral	41 (18.6)	10 (10.9)
Erythema	37 (16.8)	4 (4.3)
Chest pain	36 (16.4)	5 (5.4)
Epistaxis	36 (16.4)	9 (9.8)
Febrile neutropenia	36 (16.4)	4 (4.3)
Myalgia	35 (15.9)	2 (2.2)
Weight decreased	35 (15.9)	10 (10.9)
Abdominal pain	34 (15.5)	12 (13.0)
Pallor	34 (15.5)	7 (7.6)
Nasopharyngitis	32 (14.5)	3 (3.3)
Pitting edema	32 (14.5)	9 (9.8)
Skin lesion	32 (14.5)	8 (8.7)
Dyspnea exertional	31 (14.1)	15 (16.3)
Injection site bruising	31 (14.1)	0
Rash	31 (14.1)	9 (9.8)
Injection site reaction	30 (13.6)	0
Anxiety	29 (13.2)	3 (3.3)
Appetite decreased	28 (12.7)	8 (8.7)
Fatigue aggravated	28 (12.7)	4 (4.3)
Hypokalemia	28 (12.7)	12 (13.0)
Upper respiratory tract infection	28 (12.7)	4 (4.3)
Pruritus	27 (12.3)	11 (12.0)
Abdominal tenderness	26 (11.8)	1 (1.1)
Depression	26 (11.8)	7 (7.6)
Productive cough	25 (11.4)	4 (4.3)
Insomnia	24 (10.9)	4 (4.3)
Malaise	24 (10.9)	1 (1.1)
Pain	24 (10.9)	3 (3.3)
Pneumonia	24 (10.9)	5 (5.4)
Abdominal pain upper	23 (10.5)	3 (3.3)
Crackles lung	23 (10.5)	8 (8.7)
Sweating increased	23 (10.5)	2 (2.2)
Cardiac murmur	22 (10.0)	8 (8.7)
Rhinorrhea	22 (10.0)	2 (2.2)
Gingival bleeding	21 (9.5)	4 (4.3)
Lymphadenopathy	21 (9.5)	3 (3.3)
Herpes simplex	20 (9.1)	5 (5.4)
Hematoma	19 (8.6)	0
Night sweats	19 (8.6)	3 (3.3)
Rales	19 (8.6)	8 (8.7)
Tachycardia	19 (8.6)	6 (6.5)
Wheezing	19 (8.6)	2 (2.2)
Cellulitis	18 (8.2)	4 (4.3)
Dysuria	18 (8.2)	2 (2.2)

Preferred Term**	All Vidaza‡ (N=220)	Observation† (N=92)
At least 1 TEAE	219 (99.5)	89 (96.7)
Breath sounds decreased	17 (7.7)	1 (1.1)
Lethargy	17 (7.7)	2 (2.2)
Oral mucosal petechiae	17 (7.7)	3 (3.3)
Stomatitis	17 (7.7)	0
Urinary tract infection	17 (7.7)	5 (5.4)
Peripheral swelling	16 (7.3)	5 (5.4)
Dyspepsia	15 (6.8)	4 (4.3)
Hemorrhoids	15 (6.8)	1 (1.1)
Hypotension	15 (6.8)	2 (2.2)
Injection site pruritus	15 (6.8)	0
Transfusion reaction	15 (6.8)	0
Pleural effusion	14 (6.4)	6 (6.5)
Abdominal distension	13 (5.9)	4 (4.3)
Muscle cramps	13 (5.9)	3 (3.3)
Post procedural hemorrhage	13 (5.9)	1 (1.1)
Postnasal drip	13 (5.9)	3 (3.3)
Rhonchi	13 (5.9)	2 (2.2)
Syncope	13 (5.9)	5 (5.4)
Urticaria	13 (5.9)	1 (1.1)
Anemia aggravated	12 (5.5)	5 (5.4)
Loose stools	12 (5.5)	0
Nasal congestion	12 (5.5)	1 (1.1)
Atelectasis	11 (5.0)	2 (2.2)
Chest wall pain	11 (5.0)	0
Dry skin	11 (5.0)	1 (1.1)
Dysphagia	11 (5.0)	2 (2.2)
Dyspnea exacerbated	11 (5.0)	3 (3.3)
Hypoesthesia	11 (5.0)	1 (1.1)
Injection site granuloma	11 (5.0)	0
Injection site pigmentation changes	11 (5.0)	0
Injection site swelling	11 (5.0)	0
Mouth hemorrhage	11 (5.0)	1 (1.1)
Post procedural pain	11 (5.0)	2 (2.2)
Sinusitis	11 (5.0)	3 (3.3)
Skin nodule	11 (5.0)	1 (1.1)
Tongue ulceration	11 (5.0)	2 (2.2)

* Mean Vidaza exposure = 11.4 months. Mean time in observation arm = 6.1 months.

** Multiple reports of the same preferred terms for a patient are only counted once within each treatment group.

† Includes events from observation period only; excludes any events after crossover to Vidaza.

‡ Includes events from all patients exposed to Vidaza, including patients after crossing over from observation.

Nausea, vomiting, diarrhea, and constipation all tended to increase in incidence with increasing doses of Vidaza. Nausea, vomiting, injection site erythema, constipation, rigors, petechiae, injection site pain, dizziness, injection site bruising, anxiety, hypokalemia, insomnia, epistaxis, and rales tended to be more pronounced during the first 1-2 cycles of SC Vidaza treatment compared with later cycles of treatment. There did not appear to be any adverse events that increased in frequency over the course of treatment. There did not appear to be any relevant differences in adverse events by gender.

In clinical studies of either SC or IV Vidaza, the following serious treatment-related adverse events occurring at a rate of <5% (not described in Table 4) were reported:

Blood and lymphatic system disorders: agranulocytosis, bone marrow depression, splenomegaly.

Cardiac disorders: atrial fibrillation, cardiac failure, cardiac failure congestive, cardio-respiratory arrest, congestive cardiomyopathy.

Gastrointestinal disorders: diverticulitis, gastrointestinal hemorrhage, melena, perirectal abscess.

General disorders and administration site conditions: catheter site hemorrhage, general physical health deterioration, systemic inflammatory response syndrome.

Hepatobiliary disorders: cholecystitis.

Immune system disorders: anaphylactic shock, hypersensitivity.

Infections and infestations: abscess limb, bacterial infection, blastomycosis, injection site infection, Klebsiella sepsis, pharyngitis streptococcal, pneumonia Klebsiella, sepsis, Staphylococcal bacteremia, Staphylococcal infection, toxoplasmosis.

Metabolism and nutrition disorders: dehydration.

Musculoskeletal and connective tissue disorders: bone pain aggravated, muscle weakness, neck pain.

Neoplasms benign, malignant and unspecified: leukemia cutis.

Nervous system disorders: convulsions, intracranial hemorrhage.

Psychiatric disorders: confusion.

Renal and urinary disorders: hematuria, loin pain, renal failure.

Respiratory, thoracic and mediastinal disorders: hemoptysis, lung infiltration, pneumonitis, respiratory distress.

Skin and subcutaneous tissue disorders: pyoderma gangrenosum, rash pruritic, skin induration.

Surgical and medical procedures: cholecystectomy.

Vascular disorders: orthostatic hypotension.

OVERDOSAGE

One case of overdose with Vidaza was reported during clinical trials. A patient experienced diarrhea, nausea, and vomiting after receiving a single IV dose of approximately 290 mg/m^2, almost 4 times the recommended starting dose. The events resolved without sequelae, and the correct dose was resumed the following day. In the event of overdosage, the patient should be monitored with appropriate blood counts and should receive supportive treatment, as necessary. There is no known specific antidote for Vidaza overdosage.

DOSAGE AND ADMINISTRATION

The recommended starting dose is 75 mg/m^2 subcutaneously, daily for seven days, every four weeks. Patients should be premedicated for nausea and vomiting. The dose may be increased to 100 mg/m^2 if no beneficial effect is seen after two treatment cycles and if no toxicity other than nausea and vomiting has occurred. It is recommended that patients be treated for a minimum of 4 cycles. However, complete or partial response may require more than 4 treatment cycles. Treatment may be continued as long as the patient continues to benefit.

Patients should be monitored for hematologic response and renal toxicities (see **PRECAUTIONS)**, and dosage delay or reduction as described below may be necessary.

Dosage Adjustment Based on Hematology Laboratory Values:

- For patients with baseline (start of treatment) WBC ≥3.0 x10^9/L, ANC ≥1.5 x10^9/L, and platelets ≥75.0 x10^9/L, adjust the dose as follows, based on nadir counts for any given cycle:

Nadir Counts		% Dose in the Next Course
ANC (x10^9/L)	Platelets (x10^9/L)	
<0.5	<25.0	50%
0.5 –1.5	25.0-50.0	67%
>1.5	>50.0	100%

- For patients whose baseline counts are WBC <3.0 x10^9/L, ANC<1.5 x10^9/L, or platelets <75.0 x10^9/L, dose adjustments should be based on nadir counts and bone marrow biopsy cellularity at the time of the nadir as noted below, unless there is clear improvement in differentiation (percentage of mature granulocytes is higher and ANC is higher than at

onset of that course) at the time of the next cycle, in which case the dose of the current treatment should be continued.

WBC or Platelet Nadir % decrease in counts from baseline	Bone Marrow Biopsy Cellularity at Time of Nadir (%)		
	30-60	15-30	<15
	% Dose in the Next Course		
50 - 75	100	50	33
> 75	75	50	33

If a nadir as defined in the table above has occurred, the next course of treatment should be given 28 days after the start of the preceding course, provided that both the WBC and the platelet counts are >25% above the nadir and rising. If a >25% increase above the nadir is not seen by day 28, counts should be reassessed every 7 days. If a 25% increase is not seen by day 42, then the patient should be treated with 50% of the scheduled dose.

Dosage Adjustment Based on Renal Function and Serum Electrolytes: If unexplained reductions in serum bicarbonate levels to less than 20mEq/L occur, the dosage should be reduced by 50% on the next course. Similarly, if unexplained elevations of BUN or serum creatinine occur, the next cycle should be delayed until values return to normal or baseline and the dose should be reduced by 50% on the next treatment course (see **PRECAUTIONS** section).

Use in Geriatric Patients: Azacitidine and its metabolites are known to be substantially excreted by the kidney, and the risk of toxic reactions to this drug may be greater in patients with impaired renal function. Because elderly patients are more likely to have decreased renal function, care should be taken in dose selection, and it may be useful to monitor renal function (see **PRECAUTIONS** section).

Preparation of Vidaza

Vidaza is a cytotoxic drug and, as with other potentially toxic compounds, caution should be exercised when handling and preparing Vidaza suspensions. Please refer to **Handling and Disposal** section.

If reconstituted Vidaza comes into contact with the skin, immediately and thoroughly wash with soap and water. If it comes into contact with mucous membranes, flush thoroughly with water.

Vidaza should be reconstituted aseptically with 4 mL sterile water for injection. The diluent should be injected slowly into the vial. The vial should be inverted 2-3 times and gently rotated until a uniform suspension is achieved. The suspension will be cloudy. The resulting suspension will contain azacitidine 25 mg/mL.

Preparation for Immediate Administration: Doses greater than 4 mL should be divided equally into two syringes. The product may be held at room temperature for up to 1 hour, but must be administered within 1 hour after reconstitution.

Preparation for Delayed Administration: The reconstituted product may be kept in the vial or drawn into a syringe. Doses greater than 4 mL should be divided equally into two syringes. The product must be refrigerated immediately, and may be held under refrigerated conditions (2°C - 8°C, 36°F- 46°F) for up to 8 hours. After removal from refrigerated conditions, the suspension may be allowed to equilibrate to room temperature for up to 30 minutes prior to administration.

Administration

To provide a homogeneous suspension, the contents of the syringe must be re-suspended by inverting the syringe 2-3 times and gently rolling the syringe between the palms for 30 seconds immediately prior to administration.

Vidaza is administered subcutaneously. Doses greater than 4 mL should be divided equally into 2 syringes and injected into 2 separate sites. Rotate sites for each injection (thigh, abdomen, or upper arm). New injections should be given at least one inch from an old site and never into areas were the site is tender, bruised, red, or hard.

Stability

Reconstituted Vidaza may be stored for up to 1 hour at 25°C (77°F) or for up to 8 hours between 2 and 8°C (36 and 46°F). The Vidaza vial is single-use and does not contain any preservatives. Unused portions of each vial should be discarded properly. See **Handling and Disposal.** Do not save any unused portions for later administration.

HOW SUPPLIED

Vidaza (azacitidine for injectable suspension) is supplied as a lyophilized powder in 100 mg single-use vials packaged in cartons of 1 vial (NDC 67211-102-01).

Storage

Store unreconstituted vials at 25° C (77° F); excursions permitted to 15°-30° C (59°-86° F) (See USP Controlled Room Temperature).

Handling and Disposal

Procedures for proper handling and disposal of anticancer drugs should be applied. Several guidelines on this subject have been published[1-8]. There is no general agreement that all of the procedures recommended in the guidelines are necessary or appropriate.

REFERENCES

1. ONS Clinical Practice Committee. Cancer Chemotherapy Guidelines and Recommendations for Practice. Pittsburgh, Pa: Oncology Nursing Society; 1999:32-41.
2. Recommendations for the safe handling of parenteral antineoplastic drugs. Washington, DC: Division of Safety, National Institutes of Health; 1983. US Dept of Health and Human Services, Public Health Service publication NIH 83-2621.
3. AMA Council on Scientific Affairs. Guidelines for handling parenteral antineoplastics. *JAMA*. 1985;253:1590-1592.
4. National Study Commission on Cytotoxic Exposure. Recommendations for handling cytotoxic agents. 1987. Available from Louis P. Jeffrey, Chairman, National Study Commission on Cytotoxic Exposure. Massachusetts College of Pharmacy and Allied Health Sciences, 179 Longwood Avenue, Boston, MA 02115.
5. Clinical Oncological Society of Australia. Guidelines and recommendations for safe handling of antineoplastic agents. *Med J Australia*. 1983;1:426-428.
6. Jones RB, Frank R, Mass T. Safe handling of chemotherapeutic agents: a report from the Mount Sinai Medical Center. *CA-A Cancer J for Clin*. 1983;33:258-263.
7. American Society of Hospital Pharmacists. ASHP technical assistance bulletin on handling cytotoxic and hazardous drugs. *Am J Hosp Pharm*. 1990;47:1033-1049.
8. Controlling Occupational Exposure to Hazardous Drugs. (OSHA Work-Practice Guidelines). *Am J Health-Syst Pharm*. 1996;53:1669-1685.

Manufactured for: Pharmion Corporation
Boulder, CO 80301

Manufactured by: Ben Venue Laboratories, Inc.
Bedford, OH 44146

Edition Date: May 18, 2004

HIGHLIGHTS OF PRESCRIBING INFORMATION

These highlights do not include all the information needed to use VIDAZA safely and effectively. See full prescribing information for VIDAZA.

VIDAZA (azacitidine for injection) for SC or IV use
Initial U.S. Approval: 2004

INDICATIONS AND USAGE

VIDAZA is a nucleoside metabolic inhibitor indicated for the treatment of patients with the following FAB myelodysplastic syndrome (MDS) subtypes: Refractory anemia (RA) or refractory anemia with ringed sideroblasts (RARS) (if accompanied by neutropenia or thrombocytopenia or requiring transfusions), refractory anemia with excess blasts (RAEB), refractory anemia with excess blasts in transformation (RAEB-T), and chronic myelomonocytic leukemia (CMMoL). (1)

DOSAGE AND ADMINISTRATION

- The recommended starting dose for the first treatment cycle, for all patients regardless of baseline hematology values, is VIDAZA 75 mg/m^2 daily for 7 days to be administered by subcutaneous (SC) injection or intravenous (IV) infusion. Premedicate for nausea and vomiting. (2.1)
- Repeat cycles every 4 weeks (2.2). After 2 cycles, may increase dose to 100 mg/m^2 if no beneficial effect is seen and no toxicity other than nausea and vomiting has occurred (2.2). Patients should be treated for a minimum of 4 to 6 cycles. Complete or partial response may require additional treatment cycles (2.2).
- Continue treatment as long as the patient continues to benefit (2.2).
- Patients should be monitored for hematologic response and renal toxicities, with dosage delay or reduction as appropriate (2.3, 2.4, 2.5).

DOSAGE FORMS AND STRENGTHS

- Lyophilized powder in 100 mg single-use vials (3).

CONTRAINDICATIONS

- Advanced malignant hepatic tumors (4.1).
- Hypersensitivity to azacitidine or mannitol (4.2).

WARNINGS AND PRECAUTIONS

- Anemia, neutropenia and thrombocytopenia. Perform complete blood counts (CBC) prior to each treatment cycle and as needed to monitor response and toxicity. (5.1).
- Hepatotoxicity: Use with caution in patients with severe preexisting liver impairment (5.2).
- Renal abnormalities. Monitor patients with renal impairment for toxicity since azacitidine and its metabolites are primarily excreted by the kidneys (5.3).
- Monitor liver chemistries and serum creatinine prior to initiation of therapy and with each cycle (5.4).
- VIDAZA may cause fetal harm when administered to a pregnant woman. Women of childbearing potential should be apprised of the potential hazard to a fetus. (5.5, 8.1).
- Men should be advised not to father a child while receiving VIDAZA (5.6, 13.1).

ADVERSE REACTIONS

Most common adverse reactions (>30%) by SC route are: nausea, anemia, thrombocytopenia, vomiting, pyrexia, leukopenia, diarrhea, injection site erythema, constipation, neutropenia and ecchymosis. Most common adverse reactions by IV route also included petechiae, rigors, weakness and hypokalemia (6.1).

To report SUSPECTED ADVERSE REACTIONS, contact Celgene Corporation at 1-888-423-5436 or FDA at 1-800-FDA-1088 or *www.fda.gov/medwatch*.

DRUG INTERACTIONS

- No formal clinical assessments of drug-drug interactions between VIDAZA and other agents have been conducted (7).

USE IN SPECIFIC POPULATIONS

- Nursing Mothers: Discontinue drug or nursing taking into consideration importance of drug to mother (8.3).
- Because elderly patients are more likely to have decreased renal function, it may be useful to monitor renal function (8.5).

See 17 for PATIENT COUNSELING INFORMATION.

Revised: January 2012

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FULL PRESCRIBING INFORMATION

1 INDICATIONS AND USAGE

1.1 Myelodysplastic Syndromes (MDS)

VIDAZA® is indicated for treatment of patients with the following French-American-British (FAB) myelodysplastic syndrome subtypes: refractory anemia (RA) or refractory anemia with ringed sideroblasts (if accompanied by neutropenia or thrombocytopenia or requiring transfusions), refractory anemia with excess blasts (RAEB), refractory anemia with excess blasts in transformation (RAEB-T), and chronic myelomonocytic leukemia (CMMoL).

2 DOSAGE AND ADMINISTRATION

2.1 First Treatment Cycle

The recommended starting dose for the first treatment cycle, for all patients regardless of baseline hematology laboratory values, is 75 mg/m^2 subcutaneously or intravenously, daily for 7 days. Patients should be premedicated for nausea and vomiting.

2.2 Subsequent Treatment Cycles

Cycles should be repeated every 4 weeks. The dose may be increased to 100 mg/m^2 if no beneficial effect is seen after 2 treatment cycles and if no toxicity other than nausea and vomiting has occurred. It is recommended that patients be treated for a minimum of 4 to 6 cycles. However, complete or partial response may require additional treatment cycles. Treatment may be continued as long as the patient continues to benefit.

Patients should be monitored for hematologic response and renal toxicities *[see Warnings and Precautions (5.3)]*, and dosage delay or reduction as described below may be necessary.

2.3 Dosage Adjustment Based on Hematology Laboratory Values

- For patients with baseline (start of treatment) WBC ≥3.0 x10^9/L, ANC ≥1.5 x10^9/L, and platelets ≥75.0 x10^9/L, adjust the dose as follows, based on nadir counts for any given cycle:

Nadir Counts		% Dose in the Next Course
ANC (x10^9/L)	Platelets (x10^9/L)	
<0.5	<25.0	50%
0.5–1.5	25.0-50.0	67%
>1.5	>50.0	100%

- For patients whose baseline counts are WBC <3.0 x10^9/L, ANC<1.5 x10^9/L, or platelets <75.0 x10^9/L, dose adjustments should be based on nadir counts and bone marrow biopsy cellularity at the time of the nadir as noted below, unless there is clear improvement in differentiation (percentage of mature granulocytes is higher and ANC is higher than at onset of that course) at the time of the next cycle, in which case the dose of the current treatment should be continued.

WBC or Platelet Nadir % decrease in counts from baseline	Bone Marrow Biopsy Cellularity at Time of Nadir (%)		
	30-60	15-30	<15
	% Dose in the Next Course		
50 - 75	100	50	33
>75	75	50	33

If a nadir as defined in the table above has occurred, the next course of treatment should be given 28 days after the start of the preceding course, provided that both the WBC and the platelet counts are >25% above the nadir and rising. If a >25% increase above the nadir is not seen by day 28, counts should be reassessed every 7 days. If a 25% increase is not seen by day 42, then the patient should be treated with 50% of the scheduled dose.

2.4 Dosage Adjustment Based on Renal Function and Serum Electrolytes

If unexplained reductions in serum bicarbonate levels to <20 mEq/L occur, the dosage should be reduced by 50% on the next course. Similarly, if unexplained elevations of BUN or serum creatinine occur, the next cycle should be delayed until values return to normal or baseline and the dose should be reduced by 50% on the next treatment course *[see Warnings and Precautions (5.3)]*.

2.5 Use in Geriatric Patients

Azacitidine and its metabolites are known to be substantially excreted by the kidney, and the risk of toxic reactions to this drug may be greater in patients with impaired renal function. Because elderly patients are more likely to have decreased renal function, care should be taken in dose selection, and it may be useful to monitor renal function *[see Warnings and Precautions (5.3) and Use in Specific Populations (8.5)]*.

2.6 Preparation of VIDAZA

VIDAZA is a cytotoxic drug and, as with other potentially toxic compounds, caution should be exercised when handling and preparing VIDAZA suspensions *[see How Supplied/Storage and Handling (16)]*.

If reconstituted VIDAZA comes into contact with the skin, immediately and thoroughly wash with soap and water. If it comes into contact with mucous membranes, flush thoroughly with water.

The VIDAZA vial is single-use and does not contain any preservatives. Unused portions of each vial should be discarded properly *[see How Supplied/Storage and Handling (16)]*. Do not save any unused portions for later administration.

2.7 Instructions for Subcutaneous Administration

VIDAZA should be reconstituted aseptically with 4 mL sterile water for injection. The diluent should be injected slowly into the vial. Vigorously shake or roll the vial until a uniform suspension is achieved. The suspension will be cloudy. The resulting suspension will contain azacitidine 25 mg/mL. Do not filter the suspension after reconstitution. Doing so could remove the active substance.

Preparation for Immediate Subcutaneous Administration: Doses greater than 4 mL should be divided equally into 2 syringes. The product may be held at room temperature for up to 1 hour, but must be administered within 1 hour after reconstitution.

Preparation for Delayed Subcutaneous Administration: The reconstituted product may be kept in the vial or drawn into a syringe. Doses greater than 4 mL should be divided equally into 2 syringes. The product must be refrigerated immediately, and may be held under refrigerated conditions (2°C - 8°C, 36°F - 46°F) for up to 8 hours. After removal from refrigerated conditions, the suspension may be allowed to equilibrate to room temperature for up to 30 minutes prior to administration.

Subcutaneous Administration

To provide a homogeneous suspension, the contents of the dosing syringe must be re-suspended immediately prior to administration. To re-suspend, vigorously roll the syringe between the palms until a uniform, cloudy suspension is achieved.

VIDAZA suspension is administered subcutaneously. Doses greater than 4 mL should be divided equally into 2 syringes and injected into 2 separate sites. Rotate sites for each injection (thigh, abdomen, or upper arm). New injections should be given at least one inch from an old site and never into areas where the site is tender, bruised, red, or hard.

Suspension Stability: VIDAZA reconstituted for subcutaneous administration may be stored for up to 1 hour at 25°C (77°F) or for up to 8 hours between 2°C and 8°C (36°F and 46°F).

2.8 Instructions for Intravenous Administration

Reconstitute the appropriate number of VIDAZA vials to achieve the desired dose. Reconstitute each vial with 10 mL sterile water for injection. Vigorously shake or roll the vial until all solids are dissolved. The resulting solution will contain azacitidine 10 mg/mL. The solution should be clear. Parenteral drug product should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

Withdraw the required amount of VIDAZA solution to deliver the desired dose and inject into a 50 -100 mL infusion bag of either 0.9% Sodium Chloride Injection or Lactated Ringer's Injection.

Intravenous Solution Incompatibility

VIDAZA is incompatible with 5% Dextrose solutions, Hespan, or solutions that contain bicarbonate. These solutions have the potential to increase the rate of degradation of VIDAZA and should therefore be avoided.

Intravenous Administration

VIDAZA solution is administered intravenously. Administer the total dose over a period of 10 - 40 minutes. The administration must be completed within 1 hour of reconstitution of the VIDAZA vial.

Solution Stability: VIDAZA reconstituted for intravenous administration may be stored at 25°C (77°F), but administration must be completed within 1 hour of reconstitution.

3 DOSAGE FORMS AND STRENGTHS

VIDAZA (azacitidine for injection) is supplied as lyophilized powder in 100 mg single-use vials.

4 CONTRAINDICATIONS

4.1 Advanced Malignant Hepatic Tumors

VIDAZA is contraindicated in patients with advanced malignant hepatic tumors *[see Warnings and Precautions (5.2)]*.

4.2 Hypersensitivity to Azacitidine or Mannitol

VIDAZA is contraindicated in patients with a known hypersensitivity to azacitidine or mannitol.

5 WARNINGS AND PRECAUTIONS

5.1 Anemia, Neutropenia and Thrombocytopenia

Treatment with VIDAZA is associated with anemia, neutropenia and thrombocytopenia. Complete blood counts should be performed as needed to monitor response and toxicity, but at a minimum, prior to each dosing cycle. After administration of the recommended dosage for the first cycle, dosage for subsequent cycles should be reduced or delayed based on nadir counts and hematologic response *[see Dosage and Administration (2.3)]*.

5.2 Severe Pre-existing Hepatic Impairment

Because azacitidine is potentially hepatotoxic in patients with severe pre-existing hepatic impairment, caution is needed in patients with liver disease. Patients with extensive tumor burden due to metastatic disease have been reported to experience progressive hepatic coma and death during azacitidine treatment, especially in such patients with baseline albumin <30 g/L. Azacitidine is contraindicated in patients with advanced malignant hepatic tumors *[see Contraindications (4.1)]*.

Safety and effectiveness of VIDAZA in patients with MDS and hepatic impairment have not been studied as these patients were excluded from the clinical trials.

5.3 Renal Abnormalities
Renal abnormalities ranging from elevated serum creatinine to renal failure and death have been reported in patients treated with intravenous azacitidine in combination with other chemotherapeutic agents for nonMDS conditions. In addition, renal tubular acidosis, defined as a fall in serum bicarbonate to <20 mEq/L in association with an alkaline urine and hypokalemia (serum potassium <3 mEq/L) developed in 5 patients with CML treated with azacitidine and etoposide. If unexplained reductions in serum bicarbonate <20 mEq/L or elevations of BUN or serum creatinine occur, the dosage should be reduced or held *[see Dosage and Administration (2.4)]*.

Patients with renal impairment should be closely monitored for toxicity since azacitidine and its metabolites are primarily excreted by the kidneys *[see Dosage and Administration (2.4, 2.5)]*.

Safety and effectiveness of VIDAZA in patients with MDS and renal impairment have not been studied as these patients were excluded from the clinical trials.

5.4 Monitoring Laboratory Tests
Complete blood counts should be performed as needed to monitor response and toxicity, but at a minimum, prior to each cycle. Liver chemistries and serum creatinine should be obtained prior to initiation of therapy.

5.5 Use in Pregnancy
VIDAZA may cause fetal harm when administered to a pregnant woman. Azacitidine caused congenital malformations in animals. Women of childbearing potential should be advised to avoid pregnancy during treatment with VIDAZA. There are no adequate and well-controlled studies in pregnant women using VIDAZA. If this drug is used during pregnancy or if a patient becomes pregnant while taking this drug, the patient should be apprised of the potential hazard to the fetus *[see Use in Specific Populations (8.1)]*.

5.6 Use in Males
Men should be advised to not father a child while receiving treatment with VIDAZA. In animal studies, pre-conception treatment of male mice and rats resulted in increased embryofetal loss in mated females *[see Nonclinical Toxicology (13)]*.

6 ADVERSE REACTIONS

6.1 Overview
Adverse Reactions Described in Other Labeling Sections: anemia, neutropenia, thrombocytopenia, elevated serum creatinine, renal failure, renal tubular acidosis, hypokalemia, hepatic coma *[see Warnings and Precautions (5.1, 5.2, 5.3)]*.

Most Commonly Occurring Adverse Reactions (SC or IV Route): nausea, anemia, thrombocytopenia, vomiting, pyrexia, leukopenia, diarrhea, injection site erythema, constipation, neutropenia, ecchymosis. The most common adverse reactions by IV route also included petechiae, rigors, weakness and hypokalemia.

Adverse Reactions Most Frequently (>2%) Resulting in Clinical Intervention (SC or IV Route):
Discontinuation: leukopenia, thrombocytopenia, neutropenia.
Dose Held: leukopenia, neutropenia, thrombocytopenia, pyrexia, pneumonia, febrile neutropenia.
Dose Reduced: leukopenia, neutropenia, thrombocytopenia.

6.2 Adverse Reactions in Clinical Trials
Because clinical trials are conducted under widely varying conditions, adverse reaction rates observed in the clinical trials of a drug cannot be directly compared to rates in the clinical trials of another drug and may not reflect the rates observed in practice.

The data described below reflect exposure to VIDAZA in 443 MDS patients from 4 clinical studies. Study 1 was a supportive-care controlled trial (SC administration), Studies 2 and 3 were single arm studies (one with SC administration and one with IV administration), and Study 4 was an international randomized trial (SC administration) *[see Clinical Studies (14)]*.

In Studies 1, 2 and 3, a total of 268 patients were exposed to VIDAZA, including 116 exposed for 6 cycles (approximately 6 months) or more and 60 exposed for greater than 12 cycles (approximately one year). VIDAZA was studied primarily in supportive-care controlled and uncontrolled trials (n=150 and n=118, respectively). The population in the subcutaneous studies (n=220) was 23 to 92 years old (mean 66.4 years), 68% male, and 94% white, and had MDS or AML. The population in the IV study (n=48) was 35 to 81 years old (mean 63.1 years), 65% male, and 100% white. Most patients received average daily doses between 50 and 100 mg/m^2.

In Study 4, a total of 175 patients with higher-risk MDS (primarily RAEB and RAEB-T subtypes) were exposed to VIDAZA. Of these patients, 119 were exposed for 6 or more cycles, and 63 for at least 12 cycles. The mean age of this population was 68.1 years (ranging from 42 to 83 years), 74% were male, and 99% were white. Most patients received daily VIDAZA doses of 75 mg/m^2.

Table 1 presents adverse reactions occurring in at least 5% of patients treated with VIDAZA (SC) in Studies 1 and 2. It is important to note that duration of exposure was longer for the VIDAZA-treated group than for the observation group: patients received VIDAZA for a mean of 11.4 months while mean time in the observation arm was 6.1 months.

Table 1: Most Frequently Observed Adverse Reactions (≥5.0% in All SC VIDAZA Treated Patients; Studies 1 and 2)

	Number (%) of Patients	
System Organ Class Preferred Term[a]	**All VIDAZA[b] (N=220)**	**Observation[c] (N=92)**
Blood and lymphatic system disorders		
Anemia	153 (69.5)	59 (64.1)
Anemia aggravated	12 (5.5)	5 (5.4)
Febrile neutropenia	36 (16.4)	4 (4.3)
Leukopenia	106 (48.2)	27 (29.3)
Neutropenia	71 (32.3)	10 (10.9)
Thrombocytopenia	144 (65.5)	42 (45.7)
Gastrointestinal disorders		
Abdominal tenderness	26 (11.8)	1 (1.1)
Constipation	74 (33.6)	6 (6.5)
Diarrhea	80 (36.4)	13 (14.1)
Gingival bleeding	21 (9.5)	4 (4.3)
Loose stools	12 (5.5)	0
Mouth hemorrhage	11 (5.0)	1 (1.1)
Nausea	155 (70.5)	16 (17.4)
Stomatitis	17 (7.7)	0
Vomiting	119 (54.1)	5 (5.4)
General disorders and administration site conditions		
Chest pain	36 (16.4)	5 (5.4)
Injection site bruising	31 (14.1)	0
Injection site erythema	77 (35.0)	0
Injection site granuloma	11 (5.0)	0
Injection site pain	50 (22.7)	0
Injection site pigmentation changes	11 (5.0)	0
Injection site pruritus	15 (6.8)	0
Injection site reaction	30 (13.6)	0
Injection site swelling	11 (5.0)	0
Lethargy	17 (7.7)	2 (2.2)
Malaise	24 (10.9)	1 (1.1)
Pyrexia	114 (51.8)	28 (30.4)
Infections and infestations		
Nasopharyngitis	32 (14.5)	3 (3.3)
Pneumonia	24 (10.9)	5 (5.4)
Upper respiratory tract infection	28 (12.7)	4 (4.3)
Injury, poisoning, and procedural complications		
Post procedural hemorrhage	13 (5.9)	1 (1.1)
Metabolism and nutrition disorders		
Anorexia	45 (20.5)	6 (6.5)
Musculoskeletal and connective tissue disorders		
Arthralgia	49 (22.3)	3 (3.3)
Chest wall pain	11 (5.0)	0
Myalgia	35 (15.9)	2 (2.2)
Nervous system disorders		
Dizziness	41 (18.6)	5 (5.4)
Headache	48 (21.8)	10 (10.9)
Psychiatric disorders		
Anxiety	29 (13.2)	3 (3.3)
Insomnia	24 (10.9)	4 (4.3)
Respiratory, thoracic and mediastinal disorders		
Dyspnea	64 (29.1)	11 (12.0)

continued

Table 1: Most Frequently Observed Adverse Reactions (≥5.0% in All SC VIDAZA Treated Patients; Studies 1 and 2)

System Organ Class Preferred Term[a]	Number (%) of Patients	
	All VIDAZA[b] (N=220)	Observation[c] (N=92)
Skin and subcutaneous tissue disorders		
Dry skin	11 (5.0)	1 (1.1)
Ecchymosis	67 (30.5)	14 (15.2)
Erythema	37 (16.8)	4 (4.3)
Rash	31 (14.1)	9 (9.8)
Skin nodule	11 (5.0)	1 (1.1)
Urticaria	13 (5.9)	1 (1.1)
Vascular disorders		
Hematoma	19 (8.6)	0
Hypotension	15 (6.8)	2 (2.2)
Petechiae	52 (23.6)	8 (8.7)

[a] Multiple terms of the same preferred terms for a patient are only counted once within each treatment group.
[b] Includes adverse reactions from all patients exposed to VIDAZA, including patients after crossing over from observations.
[c] Includes adverse reactions from observation period only; excludes any adverse events after crossover to VIDAZA.

Table 2 presents adverse reactions occurring in at least 5% of patients treated with VIDAZA in Study 4. Similar to Studies 1 and 2 described above, duration of exposure to treatment with VIDAZA was longer (mean 12.2 months) compared with best supportive care (mean 7.5 months).

Table 2: Most Frequently Observed Adverse Reactions (≥5.0% in the VIDAZA Treated Patients and the Percentage with NCI CTC Grade 3/4 Reactions; Study 4)

System Organ Class Preferred Term[a]	Number (%) of Patients			
	Any Grade		Grade 3/4	
	VIDAZA (N=175)	Best Supportive Care Only (N=102)	VIDAZA (N=175)	Best Supportive Care Only (N=102)
Blood and lymphatic system disorders				
Anemia	90 (51.4)	45 (44.1)	24 (13.7)	9 (8.8)
Febrile neutropenia	24 (13.7)	10 (9.8)	22 (12.6)	7 (6.9)
Leukopenia	32 (18.3)	2 (2.0)	26 (14.9)	1 (1.0)
Neutropenia	115 (65.7)	29 (28.4)	107 (61.1)	22 (21.6)
Thrombocytopenia	122 (69.7)	35 (34.3)	102 (58.3)	29 (28.4)
Gastrointestinal disorders				
Abdominal pain	22 (12.6)	7 (6.9)	7 (4.0)	0
Constipation	88 (50.3)	8 (7.8)	2 (1.1)	0
Dyspepsia	10 (5.7)	2 (2.0)	0	0
Nausea	84 (48.0)	12 (11.8)	3 (1.7)	0
Vomiting	47 (26.9)	7 (6.9)	0	0
General disorders and administration site conditions				
Fatigue	42 (24.0)	12 (11.8)	6 (3.4)	2 (2.0)
Injection site bruising	9 (5.1)	0	0	0
Injection site erythema	75 (42.9)	0	0	0
Injection site hematoma	11 (6.3)	0	0	0
Injection site induration	9 (5.1)	0	0	0
Injection site pain	33 (18.9)	0	0	0
Injection site rash	10 (5.7)	0	0	0
Injection site reaction	51 (29.1)	0	1 (0.6)	0
Pyrexia	53 (30.3)	18 (17.6)	8 (4.6)	1 (1.0)
Infections and Infestations				
Rhinitis	10 (5.7)	1 (1.0)	0	0
Upper respiratory tract infection	16 (9.1)	4 (3.9)	3 (1.7)	0
Urinary tract infection	15 (8.6)	3 (2.9)	3 (1.7)	0

continued

Table 2: Most Frequently Observed Adverse Reactions (≥5.0% in the VIDAZA Treated Patients and the Percentage with NCI CTC Grade 3/4 Reactions; Study 4)

System Organ Class Preferred Term[a]	Number (%) of Patients			
	Any Grade		Grade 3/4	
	VIDAZA (N=175)	Best Supportive Care Only (N=102)	VIDAZA (N=175)	Best Supportive Care Only (N=102)
Investigations				
Weight decreased	14 (8.0)	0	1 (0.6)	0
Metabolism and nutrition disorders				
Hypokalemia	11 (6.3)	3 (2.9)	3 (1.7)	3 (2.9)
Nervous system disorders				
Lethargy	13 (7.4)	2 (2.0)	0	1 (1.0)
Psychiatric disorders				
Anxiety	9 (5.1)	1 (1.0)	0	0
Insomnia	15 (8.6)	3 (2.9)	0	0
Renal and urinary disorders				
Hematuria	11 (6.3)	2 (2.0)	4 (2.3)	1 (1.0)
Respiratory, thoracic and mediastinal disorders				
Dyspnea	26 (14.9)	5 (4.9)	6 (3.4)	2 (2.0)
Dyspnea exertional	9 (5.1)	1 (1.0)	0	0
Pharyngolaryngeal pain	11 (6.3)	3 (2.9)	0	0
Skin and subcutaneous tissue disorders				
Erythema	13 (7.4)	3 (2.9)	0	0
Petechiae	20 (11.4)	4 (3.9)	2 (1.1)	0
Pruritus	21 (12.0)	2 (2.0)	0	0
Rash	18 (10.3)	1 (1.0)	0	0
Vascular disorders				
Hypertension	15 (8.6)	4 (3.9)	2 (1.1)	2 (2.0)

[a] Multiple reports of the same preferred term from a patient were only counted once within each treatment.

In Studies 1, 2 and 4 with SC administration of VIDAZA, adverse reactions of neutropenia, thrombocytopenia, anemia, nausea, vomiting, diarrhea, constipation, and injection site erythema/reaction tended to increase in incidence with higher doses of VIDAZA. Adverse reactions that tended to be more pronounced during the first 1 to 2 cycles of SC treatment compared with later cycles included thrombocytopenia, neutropenia, anemia, nausea, vomiting, injection site erythema/pain/bruising/reaction, constipation, petechiae, dizziness, anxiety, hypokalemia, and insomnia. There did not appear to be any adverse reactions that increased in frequency over the course of treatment.

Overall, adverse reactions were qualitatively similar between the IV and SC studies. Adverse reactions that appeared to be specifically associated with the IV route of administration included infusion site reactions (e.g. erythema or pain) and catheter site reactions (e.g. infection, erythema, or hemorrhage).

In clinical studies of either SC or IV VIDAZA, the following serious adverse reactions occurring at a rate of < 5% (and not described in Tables 1 or 2) were reported:

Blood and lymphatic system disorders: agranulocytosis, bone marrow failure, pancytopenia splenomegaly.

Cardiac disorders: atrial fibrillation, cardiac failure, cardiac failure congestive, cardio-respiratory arrest, congestive cardiomyopathy.

Eye disorders: eye hemorrhage

Gastrointestinal disorders: diverticulitis, gastrointestinal hemorrhage, melena, perirectal abscess.

General disorders and administration site conditions: catheter site hemorrhage, general physical health deterioration, systemic inflammatory response syndrome.

Hepatobiliary disorders: cholecystitis.

Immune system disorders: anaphylactic shock, hypersensitivity.

Infections and infestations: abscess limb, bacterial infection, cellulitis, blastomycosis, injection site infection, Klebsiella sepsis, neutropenic sepsis, pharyngitis streptococcal, pneumonia Klebsiella, sepsis, septic shock, Staphylococcal bacteremia, Staphylococcal infection, toxoplasmosis.

Metabolism and nutrition disorders: dehydration.

Musculoskeletal and connective tissue disorders: bone pain aggravated, muscle weakness, neck pain.

Neoplasms benign, malignant and unspecified: leukemia cutis.

Nervous system disorders: cerebral hemorrhage, convulsions, intracranial hemorrhage.

Renal and urinary disorders: loin pain, renal failure.

Respiratory, thoracic and mediastinal disorders: hemoptysis, lung infiltration, pneumonitis, respiratory distress.

Skin and subcutaneous tissue disorders: pyoderma gangrenosum, rash pruritic, skin induration.

Surgical and medical procedures: cholecystectomy.

Vascular disorders: orthostatic hypotension.

6.3 Postmarketing Experience

The following adverse reactions have been identified during postmarketing use of VIDAZA. Because these reactions are reported voluntarily from a population of uncertain size, it is not always possible to reliably estimate their frequency or establish a causal relationship to drug exposure.

- Interstitial lung disease
- Tumor lysis syndrome
- Injection site necrosis
- Sweet's syndrome (acute febrile neutrophilic dermatosis)

7 DRUG INTERACTIONS

No formal clinical assessments of drug-drug interactions between VIDAZA and other agents have been conducted *[see Clinical Pharmacology (12.3)]*.

8 USE IN SPECIFIC POPULATIONS

8.1 Pregnancy

Pregnancy Category D *[see Warning and Precautions (5.5)]*

VIDAZA may cause fetal harm when administered to a pregnant woman. Azacitidine was teratogenic in animals. There are no adequate and well controlled studies with Vidaza in pregnant women. Women of childbearing potential should be advised to avoid pregnancy during treatment with VIDAZA. If this drug is used during pregnancy or if a patient becomes pregnant while taking this drug, the patient should be apprised of the potential hazard to the fetus.

Female partners of male patients receiving VIDAZA should not become pregnant *[see Nonclinical Toxicology (13.1)]*.

Early embryotoxicity studies in mice revealed a 44% frequency of intrauterine embryonal death (increased resorption) after a single IP (intraperitoneal) injection of 6 mg/m^2 (approximately 8% of the recommended human daily dose on a mg/m^2 basis) azacitidine on gestation day 10. Developmental abnormalities in the brain have been detected in mice given azacitidine on or before gestation day 15 at doses of ~3-12 mg/m^2 (approximately 4%-16% the recommended human daily dose on a mg/m^2 basis).

In rats, azacitidine was clearly embryotoxic when given IP on gestation days 4-8 (postimplantation) at a dose of 6 mg/m^2 (approximately 8% of the recommended human daily dose on a mg/m^2 basis), although treatment in the preimplantation period (on gestation days 1-3) had no adverse effect on the embryos. Azacitidine caused multiple fetal abnormalities in rats after a single IP dose of 3 to 12 mg/m^2 (approximately 8% the recommended human daily dose on a mg/m^2 basis) given on gestation day 9, 10, 11 or 12. In this study azacitidine caused fetal death when administered at 3-12 mg/m^2 on gestation days 9 and 10; average live animals per litter was reduced to 9% of control at the highest dose on gestation day 9. Fetal anomalies included: CNS anomalies (exencephaly/encephalocele), limb anomalies (micromelia, club foot, syndactyly, oligodactyly), and others (micrognathia, gastroschisis, edema, and rib abnormalities).

8.3 Nursing Mothers

It is not known whether azacitidine or its metabolites are excreted in human milk. Because many drugs are excreted in human milk and because of the potential for tumorigenicity shown for azacitidine in animal studies and the potential for serious adverse reactions in nursing infants from Vidaza, a decision should be made whether to discontinue nursing or to discontinue the drug, taking into consideration the importance of the drug to the mother.

8.4 Pediatric Use

Safety and effectiveness in pediatric patients have not been established.

8.5 Geriatric Use

Of the total number of patients in Studies 1, 2 and 3, 62% were 65 years and older and 21% were 75 years and older. No overall differences in effectiveness were observed between these patients and younger patients. In addition there were no relevant differences in the frequency of adverse reactions observed in patients 65 years and older compared to younger patients.

Of the 179 patients randomized to azacitidine in Study 4, 68% were 65 years and older and 21% were 75 years and older. Survival data for patients 65 years and older were consistent with overall survival results. The majority of adverse reactions occurred at similar frequencies in patients < 65 years of age and patients 65 years of age and older.

Azacitidine and its metabolites are known to be substantially excreted by the kidney, and the risk of adverse reactions to this drug may be greater in patients with impaired renal function. Because elderly patients are more likely to have decreased renal function, it may be useful to monitor renal function *[see Dosage and Administration (2.5) and Warnings and Precautions (5.3)]*.

8.6 Gender

There were no clinically relevant differences in safety and efficacy based on gender.

8.7 Race

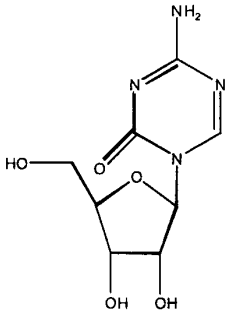
Greater than 90% of all patients in all trials were Caucasian. Therefore, no comparisons between Caucasians and non-Caucasians were possible.

10 OVERDOSAGE

One case of overdose with VIDAZA was reported during clinical trials. A patient experienced diarrhea, nausea, and vomiting after receiving a single IV dose of approximately 290 mg/m^2, almost 4 times the recommended starting dose. The events resolved without sequelae, and the correct dose was resumed the following day. In the event of overdosage, the patient should be monitored with appropriate blood counts and should receive supportive treatment, as necessary. There is no known specific antidote for VIDAZA overdosage.

11 DESCRIPTION

VIDAZA (azacitidine for injection) contains azacitidine, which is a pyrimidine nucleoside analog of cytidine. Azacitidine is 4-amino-1-β-D-ribofuranosyl-s-triazin-2(1H)-one. The structural formula is as follows:



The empirical formula is $C_8H_{12}N_4O_5$. The molecular weight is 244. Azacitidine is a white to off-white solid. Azacitidine was found to be insoluble in acetone, ethanol, and methyl ethyl ketone; slightly soluble in ethanol/water (50/50), propylene glycol, and polyethylene glycol; sparingly soluble in water, water saturated octanol, 5% dextrose in water, N-methyl-2-pyrrolidone, normal saline and 5% Tween 80 in water; and soluble in dimethylsulfoxide (DMSO).

The finished product is supplied in a sterile form for reconstitution as a suspension for subcutaneous injection or reconstitution as a solution with further dilution for intravenous infusion. Vials of VIDAZA contain 100 mg of azacitidine and 100 mg mannitol as a sterile lyophilized powder.

12 CLINICAL PHARMACOLOGY

12.1 Mechanism of Action

VIDAZA is a pyrimidine nucleoside analog of cytidine. VIDAZA is believed to exert its antineoplastic effects by causing hypomethylation of DNA and direct cytotoxicity on abnormal hematopoietic cells in the bone marrow. The concentration of azacitidine required for maximum inhibition of DNA methylation *in vitro* does not cause major suppression of DNA synthesis. Hypomethylation may restore normal function to genes that are critical for differentiation and proliferation. The cytotoxic effects of azacitidine cause the death of rapidly dividing cells, including cancer cells that are no longer responsive to normal growth control mechanisms. Non-proliferating cells are relatively insensitive to azacitidine.

12.3 Pharmacokinetics

The pharmacokinetics of azacitidine were studied in 6 MDS patients following a single 75 mg/m^2 subcutaneous (SC) dose and a single 75 mg/m^2 intravenous (IV) dose. Azacitidine is rapidly absorbed after SC administration; the peak plasma azacitidine concentration of 750 ± 403 ng/ml occurred in 0.5 hour. The bioavailability of SC azacitidine relative to IV azacitidine is approximately 89%, based on area under the curve. Mean volume of distribution following IV dosing is 76 ± 26 L. Mean apparent SC clearance is 167 ± 49 L/hour and mean half-life after SC administration is 41 ± 8 minutes.

Published studies indicate that urinary excretion is the primary route of elimination of azacitidine and its metabolites. Following IV administration of radioactive azacitidine to 5 cancer patients, the cumulative urinary excretion was 85% of the radioactive dose. Fecal excretion accounted for <1% of administered radioactivity over 3 days. Mean excretion of radioactivity in urine following SC administration of ^{14}C-azacitidine was 50%. The mean elimination half-lives of total radioactivity (azacitidine and its metabolites) were similar after IV and SC administrations, about 4 hours.

Special Populations

The effects of renal or hepatic impairment, gender, age, or race on the pharmacokinetics of azacitidine have not been studied *[see Dosage and Administration (2.4), Contraindications (4.1) and Warnings and Precautions (5.2, 5.3)]*.

Drug-Drug Interactions

No formal clinical drug interaction studies with azacitidine have been conducted.

An *in vitro* study of azacitidine incubation in human liver fractions indicated that azacitidine may be metabolized by the liver. Whether azacitidine metabolism may be affected by known microsomal enzyme inhibitors or inducers has not been studied.

An *in vitro* study with cultured human hepatocytes indicated that azacitidine at concentrations up to 100 µM (IV Cmax = 10.6 µM) does not cause any inhibition of CYP2B6 and CYP2C8. The potential of azacitidine to inhibit other cytochrome P450 (CYP) enzymes is not known.

In vitro studies with human cultured hepatocytes indicate that azacitidine at concentrations of 1.0 µM to 100 µM does not induce CYP 1A2, 2C19, or 3A4/5.

13 NONCLINICAL TOXICOLOGY

13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility

The potential carcinogenicity of azacitidine was evaluated in mice and rats. Azacitidine induced tumors of the hematopoietic system in female mice at 2.2 mg/kg (6.6 mg/m^2, approximately 8% the recommended human daily dose on a mg/m^2 basis) administered IP three times per week for 52 weeks. An increased incidence of tumors in the lymphoreticular system, lung, mammary gland, and skin was seen in mice treated with azacitidine IP at 2.0 mg/kg (6.0 mg/m^2, approximately 8% the recommended human daily dose on a mg/m^2 basis) once a week for 50 weeks. A tumorigenicity study in rats dosed twice weekly at 15 or 60 mg/m^2 (approximately 20-80% the recommended human daily dose on a mg/m^2 basis) revealed an increased incidence of testicular tumors compared with controls.

The mutagenic and clastogenic potential of azacitidine was tested in *in vitro* bacterial systems Salmonella typhimurium strains TA100 and several strains of trpE8, Escherichia coli strains WP14 Pro, WP3103P, WP3104P, and CC103; in *in vitro* forward gene mutation assay in mouse lymphoma cells and human lymphoblast cells; and in an *in vitro* micronucleus assay in mouse L5178Y lymphoma cells and Syrian hamster embryo cells. Azacitidine was mutagenic in bacterial and mammalian cell systems. The clastogenic effect of azacitidine was shown by the induction of micronuclei in L5178Y mouse cells and Syrian hamster embryo cells.

Administration of azacitidine to male mice at 9.9 mg/m^2 (approximately 9% the recommended human daily dose on a mg/m^2 basis) daily for 3 days prior to mating with untreated female mice resulted in decreased fertility and loss of offspring during subsequent embryonic and postnatal development. Treatment of male rats 3 times per week for 11 or 16 weeks at doses of 15-30 mg/m^2 (approximately 20-40%, the recommended human daily dose on a mg/m^2 basis) resulted in decreased weight of the testes and epididymides, and decreased sperm counts accompanied by decreased pregnancy rates and increased loss of embryos in mated females. In a related study, male rats treated for 16 weeks at 24 mg/m^2 resulted in an increase in abnormal embryos in mated females when examined on day 2 of gestation.

14 CLINICAL STUDIES

Myelodysplastic Syndromes (MDS)

Study 1 was a randomized, open-label, controlled trial carried out in 53 U.S. sites compared the safety and efficacy of subcutaneous VIDAZA plus supportive care with supportive care alone ("observation") in patients with any of the five FAB subtypes of myelodysplastic syndromes (MDS): refractory anemia (RA), RA with ringed sideroblasts (RARS), RA with excess blasts (RAEB), RAEB in transformation (RAEB-T), and chronic myelomonocytic leukemia (CMMoL). RA and RARS patients were included if they met one or more of the following criteria: required packed RBC transfusions; had platelet counts ≤50.0 x 10^9/L; required platelet transfusions; or were neutropenic (ANC <1.0 x 10^9/L) with infections requiring treatment with antibiotics. Patients with acute myelogenous leukemia (AML) were not intended to be included. Supportive care allowed in this study included blood transfusion products, antibiotics, antiemetics, analgesics and antipyretics. The use of hematopoeitic growth factors was prohibited. Baseline patient and disease characteristics are summarized in Table 3; the 2 groups were similar.

VIDAZA was administered at a subcutaneous dose of 75 mg/m^2 daily for 7 days every 4 weeks. The dose was increased to 100 mg/m^2 if no beneficial effect was seen after 2 treatment cycles. The dose was decreased and/or delayed based on hematologic response or evidence of renal toxicity. Patients in the observation arm were allowed by protocol to cross over to VIDAZA if they had increases in bone marrow blasts, decreases in hemoglobin, increases in red cell transfusion requirements, or decreases in platelets, or if they required a platelet transfusion or developed a clinical infection requiring treatment with antibiotics. For purposes of assessing efficacy, the primary endpoint was response rate (as defined in Table 4).

Of the 191 patients included in the study, independent review (adjudicated diagnosis) found that 19 had the diagnosis of AML at baseline. These patients were excluded from the primary analysis of response rate, although they were included in an intent-to-treat (ITT) analysis of all patients randomized. Approximately 55% of the patients randomized to observation crossed over to receive VIDAZA treatment.

Table 3. Baseline Demographics and Disease Characteristics

	VIDAZA (N=99)	Observation (N=92)
Gender (n%)		
Male	72 (72.7)	60 (65.2)
Female	27 (27.3)	32 (34.8)

continued

Table 3. Baseline Demographics and Disease Characteristics

	VIDAZA (N=99)	Observation (N=92)
Race (n%)		
White	93 (93.9)	85 (92.4)
Black	1 (1.0)	1 (1.1)
Hispanic	3 (3.0)	5 (5.4)
Asian/Oriental	2 (2.0)	1 (1.1)
Age (years)		
N	99	91
Mean ± SD	67.3 ± 10.39	68.0 ± 10.23
Range	31 - 92	35 - 88
Adjudicated MDS diagnosis at study entry (n%)		
RA	21 (21.2)	18 (19.6)
RARS	6 (6.1)	5 (5.4)
RAEB	38 (38.4)	39 (42.4)
RAEB-T	16 (16.2)	14 (15.2)
CMMoL	8 (8.1)	7 (7.6)
AML	10 (10.1)	9 (9.8)
Transfusion product used in 3 months before study entry (n%)		
Any transfusion product	70 (70.7)	59 (64.1)
Blood cells, packed human	66 (66.7)	55 (59.8)
Platelets, human blood	15 (15.2)	12 (13.0)
Hetastarch	0 (0.0)	1 (1.1)
Plasma protein fraction	1 (1.0)	0 (0.0)
Other	2 (2.0)	2 (2.2)

Table 4. Response Criteria

		RA	RARS	RAEB	RAEB-T	CMMoL
Complete Response (CR), duration ≥4 weeks	**Marrow**	<5% blasts				
	Peripheral Blood	Normal CBC if abnormal at baseline Absence of blasts in the peripheral circulation				
Partial Response (PR), duration ≥4 weeks	**Marrow**	No marrow requirements		≥50% decrease in blasts Improvement of marrow dyspoiesis		
	Peripheral Blood	≥50% restoration in the deficit from normal levels of baseline white cells, hemoglobin and platelets if abnormal at baseline No blasts in the peripheral circulation For CMMoL, if WBC is elevated at baseline, a ≥75% reduction in the excess count over the upper limit of normal				

The overall response rate (CR + PR) of 15.7% in VIDAZA-treated patients without AML (16.2% for all VIDAZA randomized patients including AML) was statistically significantly higher than the response rate of 0% in the observation group (p<0.0001) (Table 5). The majority of patients who achieved either CR or PR had either 2 or 3 cell line abnormalities at baseline (79%; 11/14) and had elevated bone marrow blasts or were transfusion dependent at baseline. Patients responding to VIDAZA had a decrease in bone marrow blasts percentage, or an increase in platelets, hemoglobin or WBC. Greater than 90% of the responders initially demonstrated these changes by the 5th treatment cycle. All patients who had been transfusion dependent became transfusion independent during PR or CR. The mean and median duration of clinical response of PR or better was estimated as 512 and 330 days, respectively; 75% of the responding patients were still in PR or better at completion of treatment. Response occurred in all MDS subtypes as well as in patients with adjudicated baseline diagnosis of AML.

Table 5. Response Rates

	VIDAZA (N=89)	Observation Before Crossover (N=83)	
Response	**n (%)**	**n (%)**	**P value**
Overall (CR+PR)	14 (15.7)	0 (0.0)	(<0.0001)
Complete (CR)	5 (5.6)	0 (0.0)	(0.06)
Partial (PR)	9 (10.1)	0 (0.0)	–

Patients in the observation group who crossed over to receive VIDAZA treatment (47 patients) had a response rate of 12.8%.

Study 2, a multi-center, open-label, single-arm study of 72 patients with RAEB, RAEB-T, CMMoL, or AML was also carried out. Treatment with subcutaneous

VIDAZA resulted in a response rate (CR + PR) of 13.9%, using criteria similar to those described above. The mean and median duration of clinical response of PR or better was estimated as 810 and 430 days, respectively; 80% of the responding patients were still in PR or better at the time of completion of study involvement. In Study 3, another open-label, single-arm study of 48 patients with RAEB, RAEB-T, or AML, treatment with intravenous VIDAZA resulted in a response rate of 18.8%, again using criteria similar to those described above. The mean and median duration of clinical response of PR or better was estimated as 389 and 281 days, respectively; 67% of the responding patients were still in PR or better at the time of completion of treatment. Response occurred in all MDS subtypes as well as in patients with adjudicated baseline diagnosis of AML in both of these studies. VIDAZA dosage regimens in these 2 studies were similar to the regimen used in the controlled study.

Benefit was seen in patients who did not meet the criteria for PR or better, but were considered "improved." About 24% of VIDAZA-treated patients were considered improved, and about 2/3 of those lost transfusion dependence. In the observation group, only 5/83 patients met criteria for improvement; none lost transfusion dependence. In all 3 studies, about 19% of patients met criteria for improvement with a median duration of 195 days.

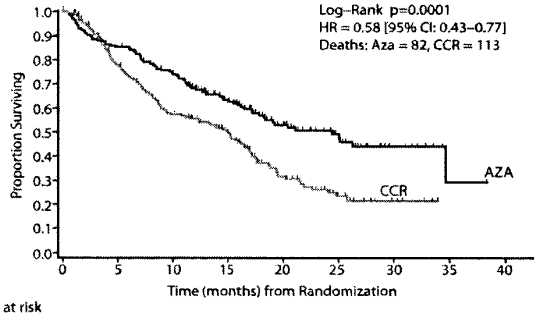
Study 4 was an international, multicenter, open-label, randomized trial in MDS patients with RAEB, RAEB-T or modified CMMoL according to FAB classification and Intermediate-2 and High risk according to IPSS classification. Of the 358 patients enrolled in the study, 179 were randomized to receive azacitidine plus best supportive care (BSC) and 179 were randomized to receive conventional care regimens (CCR) plus BSC (105 to BSC alone, 49 to low dose cytarabine and 25 to chemotherapy with cytarabine and anthracycline). The primary efficacy endpoint was overall survival.

The azacitidine and CCR groups were comparable for baseline parameters. The median age of patients was 69 years (range was 38-88 years), 98% were Caucasian, and 70% were male. At baseline, 95% of the patients were higher risk by FAB classification: RAEB (58%), RAEB-T (34%), and CMMoL (3%). By IPSS classification, 87% were higher risk: Int-2 (41%), High (47%). At baseline, 32% of patients met WHO criteria for AML.

Azacitidine was administered subcutaneously at a dose of 75 mg/m² daily for 7 consecutive days every 28 days (which constituted one cycle of therapy). Patients continued treatment until disease progression, relapse after response, or unacceptable toxicity. Azacitidine patients were treated for a median of 9 cycles (range 1 to 39), BSC only patients for a median of 7 cycles (range 1 to 26), low dose cytarabine patients for a median of 4.5 cycles (range 1 to 15), and chemotherapy with cytarabine and anthracycline patients for a median of 1 cycle (range 1 to 3, i.e. induction plus 1 or 2 consolidation cycles).

In the Intent-to-Treat analysis, patients treated with azacitidine demonstrated a statistically significant difference in overall survival as compared to patients treated with CCR (median survival of 24.5 months vs. 15.0 months; stratified log-rank p=0.0001). The hazard ratio describing this treatment effect was 0.58 (95% CI: 0.43, 0.77).

Kaplan-Meier Curve of Time to Death from Any Cause: (Intent-to-Treat Population)



# at risk									
AZA	179	152	130	85	52	30	10	1	0
CCR	179	132	95	69	32	14	5	0	0

Key: AZA = azacitidine; CCR = conventional care regimens; CI = confidence interval; HR = Hazard Ratio

Azacitidine treatment led to a reduced need for red blood cell transfusions (see Table 6). In patients treated with azacitidine who were RBC transfusion dependent at baseline and became transfusion independent, the median duration of RBC transfusion independence was 13.0 months.

Table 6. Effect of Azacitidine on RBC Transfusions in MDS Patients

Efficacy Parameter	Azacitidine plus BSC (n= 179)	Conventional Care Regimens (n= 179)
Number and percent of patients who were transfusion dependent at baseline who became transfusion independent on treatment[1]	50/111 (45.0%) (95% CI: 35.6%, 54.8%)	13/114 (11.4%) (95% CI: 6.2%, 18.7%)
Number and percent of patients who were transfusion-independent at baseline who became transfusion-dependent on treatment	10/68 (14.7%) (95% CI: 7.3%, 25.4%)	28/65 (43.1%) (95% CI: 30.9%, 56.0%)

[1] A patient was considered RBC transfusion independent during the treatment period if the patient had no RBC transfusions during any 56 consecutive days or more during the treatment period. Otherwise, the patient was considered transfusion dependent.

15 REFERENCES

1. Preventing Occupational Exposures to Antineoplastic and Other Hazardous Drugs in Health Care Settings. NIOSH Alert 2004-165.
2. OSHA Technical Manual, TED 1-0.15A, Section VI: Chapter 2. Controlling Occupational Exposure to Hazardous Drugs. OSHA, 1999. http://www.osha.gov/dts/osta/otm/otm_vi/otm_vi_2.html
3. American Society of Health-System Pharmacists. ASHP guidelines on handling hazardous drugs. Am J Health-Syst Pharm. (2006) 63:1172-1193.
4. Polovich, M., White, J. M., & Kelleher, L.O. (eds.) 2005. Chemotherapy and biotherapy guidelines and recommendations for practice (2nd. ed.) Pittsburgh, PA: Oncology Nursing Society.

16 HOW SUPPLIED/STORAGE AND HANDLING

How Supplied

VIDAZA (azacitidine for injection) is supplied as a lyophilized powder in 100 mg single-use vials packaged in cartons of 1 vial (NDC 59572-102-01).

Storage

Store unreconstituted vials at 25° C (77° F); excursions permitted to 15°-30° C (59°-86° F) (See USP Controlled Room Temperature).

Handling and Disposal

Procedures for proper handling and disposal of anticancer drugs should be applied. Several guidelines on this subject have been published.[1-4] There is no general agreement that all of the procedures recommended in the guidelines are necessary or appropriate.

17 PATIENT COUNSELING INFORMATION

Instruct patients to inform their physician about any underlying liver or renal disease.

Advise women of childbearing potential to avoid becoming pregnant while receiving treatment with VIDAZA. For nursing mothers, a decision should be made whether to discontinue nursing or to discontinue the drug, taking into consideration the importance of the drug to the mother.

Advise men not to father a child while receiving treatment with VIDAZA.

Manufactured for: Celgene Corporation
Summit, NJ 07901

Manufactured by: Ben Venue Laboratories, Inc.
Bedford, OH 44146

Or

Baxter Oncology GmbH
33790 Halle/Westfalen Germany

Or

BSP Pharmaceuticals S.r.l.
04013 Latina Scalo (Lt)
Italy

VIDPI.006 01/12

Electronic Acknowledgement Receipt

EFS ID:	15800223
Application Number:	12466213
International Application Number:	
Confirmation Number:	5370
Title of Invention:	ORAL FORMULATIONS OF CYTIDINE ANALOGS AND METHODS OF USE THEREOF
First Named Inventor/Applicant Name:	Jeffrey B. Etter
Customer Number:	84802
Filer:	Jihong Lou/Keiko Masuyama Hicks
Filer Authorized By:	Jihong Lou
Attorney Docket Number:	9516-847-999/501872-847
Receipt Date:	16-MAY-2013
Filing Date:	14-MAY-2009
Time Stamp:	20:05:35
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Transmittal Letter	9516-847-999_IDS.pdf	76133 09fcb0ee3238cc64dc282bcc3aa51da885df4810	no	1

Warnings:

Information:

2	Information Disclosure Statement (IDS) Form (SB08)	9516-847-999_ListOfReferences.pdf	36838 53df8c4c0a541070d79f2e8074ae7ccd6dc5c5b8	no	2
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
3	Foreign Reference	WO_2004082822.pdf	939883 9fc026eb8c73d6a0baeaf26d4ee17c943cc4517c	no	21
Warnings:					
Information:					
4	Foreign Reference	WO2006089290.pdf	6570785 0065d45ca2ce7d4af3c7edb330a90e05eca058c5	no	113
Warnings:					
Information:					
5	Foreign Reference	WO_2009139888.pdf	6371626 66e14a52266324c145b03bfd553b2e47b14767bc	no	130
Warnings:					
Information:					
6	Foreign Reference	WO2010059969.pdf	4591844 a0ff8734aef0ae1c20d57d4c8c2641e2979cd2de	no	77
Warnings:					
Information:					
7	Foreign Reference	WO2012135405.pdf	5587486 e1774aaf6505330962d240dc4837c3ad754aa131	no	122
Warnings:					
Information:					
8	Foreign Reference	WO2013022872.pdf	7337618 d83b51f5fc6bec2c3dc0b05fa7e37843543e1435	no	105
Warnings:					
Information:					
9	Non Patent Literature	Anonymous_clinicaltrials_2011-08-05.PDF	111270 a059cd5988576f4815191109d6ec54e1cf50131a	no	3
Warnings:					
Information:					
10	Non Patent Literature	Anonymous_clinicaltrials_2011-11-22.PDF	156604 b93b961c26559ed9d97904d03a88a5ae3b11742b	no	4

Warnings:					
Information:					
11	Non Patent Literature	Bast_2008_Abstract3500.PDF	64835 ad12c0126b100b8e6de4e8168a97c00fe4a57550	no	1
Warnings:					
Information:					
12	Non Patent Literature	Cowan_2010_71-86.PDF	1144913 af486e3dbc5189bed0b95696f63ce04ab17810a5	no	16
Warnings:					
Information:					
13	Non Patent Literature	Glaser_2007_659-671.pdf	2203459 75a3d5b4ebdbc00fabaadc6e2caf3a5e9de24483	no	13
Warnings:					
Information:					
14	Non Patent Literature	Howell_2010_2022-2044.PDF	508823 ffe8327b4adf663ea6bdbba1e2c2b4440fd8adf2	no	23
Warnings:					
Information:					
15	Non Patent Literature	Juergens_2009_InterimAnalysis.PDF	60828 ed58220b4065f599bc24b0ed97c17f063773ebf6	no	2
Warnings:					
Information:					
16	Non Patent Literature	Momparler_2005_443-451.PDF	691191 3ca2ad9584fcfc552334b159f2d7477be6d542a8	no	9
Warnings:					
Information:					
17	Non Patent Literature	Stathis_2011-1582-1590.PDF	677944 80de240380dcf1e82917b02fd2ca9d6da862923c	no	9
Warnings:					
Information:					
18	Non Patent Literature	Vidaza_TMLabel_2004.PDF	193278 09a4ee5d7769f9b6f8458e2c674aa41b2c094143	no	18
Warnings:					
Information:					
19	Non Patent Literature	Vidaza_2012_PrescribingInformation_Jan2012.PDF	351846 ca57446636a21df3367f39adad0ec0483d118c77	no	7

Warnings:	
Information:	
Total Files Size (in bytes):	37677204

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	Jeffrey B. Etter et al.	**Confirmation No.**:	5370
Application No.:	12/466,213	**Group Art Unit**:	1623
Filed:	May 14, 2009	**Examiner**:	Lawrence E. Crane
For:	ORAL FORMULATIONS OF CYTIDINE ANALOGS AND METHODS OF USE THEREOF	**Attorney Docket No.**:	9516-847-999 (501872-999847)

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT

E-FILE
Commissioner for Patents
P.O. Box 1450
Washington, D.C. 22313-1450

Sir:

Pursuant to Applicants' duty of disclosure under 37 C.F.R. §§ 1.56 and 1.97, enclosed is a list of references for the Examiner's review and consideration. These references (A48–A49, B13–B18 and C72–C82) are listed on the enclosed form entitled "List of References Cited by Applicant." Copies of B13–B18 and C72–C82 are also enclosed herewith.

Identification of the foregoing references is not to be construed as an admission of Applicants or Attorneys for Applicants that such references are available as "prior art" against the subject application. Applicants respectfully request that the Examiner review the foregoing references and make them of record by completing and returning the enclosed List of References.

No fee is believed to be due for the submission of this statement, because it is submitted after a Request for Continued Examination. However, the Director is authorized to charge any required fees to Jones Day Deposit Account No. 50-3013 (referencing CAM: 501872-999847).

Respectfully submitted,

Date May 16, 2013

/Jihong Lou/

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For: Yeah-Sil Moon (Reg. No. 52,042)
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SDI-163918v1

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/466,213	05/14/2009	Jeffrey B. Etter	9516-847-999/501872-847	5370

84802 7590 08/29/2013

JONES DAY for Celgene Corporation
222 E. 41ST. STREET
NEW YORK, NY 10017

EXAMINER	
CRANE, LAWRENCE E	
ART UNIT	PAPER NUMBER
1623	
MAIL DATE	DELIVERY MODE
08/29/2013	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)
	12/466,213	ETTER ET AL.
	Examiner	**Art Unit**
	Lawrence E. Crane	1623

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *03 August 2012*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1,3-23,31-55,57,59-61 and 72* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1,3-23,31-55,57,59-61 and 72* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☒ The drawing(s) filed on *14 May 2009* is/are: a)☒ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)	
1) ☐ Notice of References Cited (PTO-892)	4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)	5) ☐ Notice of Informal Patent Application
3) ☒ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date *09/05/2012; 05/16/2013*.	6) ☐ Other: _____.

The present application is being examined under the pre-AIA first to invent provisions.

Claims **2, 56 and 62-65** were previously cancelled, claims **24-30, 58 and 66-71** have been newly cancelled, claims **1, 6, 9, 12-23, 31, 36, 41-44, 51, 52, 57 and 61** have been amended, the Abstract has not been further amended, the disclosure has not been further amended, and new claim **72** has been added as per the amendment filed August 3, 2012. Two additional or supplemental Information Disclosure Statements (2 IDSs) filed September 5, 2012 and May 16, 2013 have been received with all cited non-U.S. patent references, annotated, and made of record.

Claims **1, 3-23, 31-55, 57, 59-61 and 72** remain in the case.

Note to applicant: when a rejection refers to a claim **X** at line y, the line number "y" is determined from the claim as previously submitted by applicant in the most recent response including ~~lines deleted by line through~~.

Claims **1, 3-23, 31-55, 57, 59-61 and 72** are rejected under 35 U.S.C. §112, first paragraph, because the specification, while being enabled for the treatment by the administration of 5-azacytidine as the sole active ingredient of a limited number of neoplastic disease conditions (see the non-prospective disclosures in Examples 1-7) and pharmaceutical compositions comprising 5-azacytidine capable of oral administration and formulated for adsorption in the stomach, does not reasonably provide enablement for either the treating of the diseases MDL and AML when a second "anti-cancer agent" is present, or pharmaceutical compositions comprising an "immediate release" capability wherein the specific excipients and/or carriers which permit this feature to be realized have not been identified with a adequately defined scope (see claims **12, 31, 57 and 72**). The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to use the invention commensurate in scope with these claims.

The fundamental issue here is whether practicing the full scope of the instant invention is possible without undue experimentation. As provided for in *In re Wands* (858 F.2d 731, 737; 8 USPQ 2d 1400, 1404 (Fed Cir. 1988) the minimum factors to be considered in determination of whether a conclusion of "undue experimentation" is appropriate are as follows:

A. The breadth of the claims: Instant claims **23-44** are directed to the treatment of "MDL" and "AML" including when a second "anti-cancer agent" is being administered, a scope of coverage which has not been enabled by the instant disclosed exemplifications. A similar criticism applies to all of the remaining pharmaceutical composition claims wherein claims **12, 57 and 72** include the second "anti-cancer agent" option.

B. The nature of the claimed subject matter: This subject has been disclosed in the introduction to this rejection and in the previous paragraph.

C. The state of the prior art: The treatment of neoplastic diseases by the administration of an effective amount of 5-azacytidine to a host in need thereof is very well known in the art as disclosed and taught by PTO-892 references **B, R**, **S**, and **T**. In addition **Redkar '046** (PTO-892 ref. **B**) at paragraph **[0181]** discloses that 5-azacytidine may be administered to a host in need thereof in combination with one or more "anti-neoplastic agents," aka "anti-cancer agents."

D. The level of one or ordinary skill: One of ordinary skill would be expected to be knowledgeable concerning the identification of anti-neoplastic agents, the pharmaceutical compositions thereof, and with the administration thereof to treat neoplastic disease conditions.

E. The level of predictability in the art: In view of the substantial and relevant teachings of both PTO-1449 and PTO-892 cited documents presently of record, teachings which disclose that 5-azacytidine has neoplastic activity. The substantial amount of highly relevant and on point prior art already of record supports the view that this art area is at least somewhat predictable.

F. The amount of direction provided by the inventor: As noted above the instant, non-prospective exemplifications appear to end at Example 7. The small number of instant exemplifications suggests that the instant disclosure has enabled only a very limited number of disease treatments wherein 5-azacytidine is administered as the active ingredient, and has provided no non-prospective exemplifications wherein 5-azacytidine has been administer together with a second anti-cancer agent. .

G. The existence of working examples: This factor is dealt within the previous paragraph.

H. The quantity of experimentation needed to make or use the invention based on the content of the disclosure has been found to be excessive for the reasons noted above, reasons effectively summarized as an insufficient number of relevant exemplifications to adequately support the scope of subject matter presently being claimed.

Applicant's arguments with respect to claims **1, 3-55, 57, 59-61 and 66-71** have been considered but are moot in view of the new grounds of rejection. This new ground of rejection was necessitated by applicant's amendments.

Claims **1, 3-23, 31-55, 57, 59-61 and 72** are rejected under 35 U.S.C. §112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

In claim **1** the term "at least one pharmaceutically acceptable excipient" appears to be inconsistent with the terms "immediate release tablet" and "immediate release capsule" because the included term "immediate release" implies the presence of "more than one excipient." In addition the claim is incomplete because the entire contents of the "tablet" and the "capsule" have only been defined functionally in the claim by the term "immediate release." The most effective way to address this issue is to define in the independent pharmaceutical composition claims the particular combination or combinations of excipients and carriers necessary to produce "immediate release" following oral ingestion.

In claim **12** the term "an additional therapeutic agent" renders the claim incomplete because the identity of the intended additional agent(s) has/have not been provided in the claim and also because the noted term lacks adequately defined metes and bounds. In addition, this claim is superfluous in view of the term "comprising" in claim **1**, a term of art which means that any pharmaceutical composition claim invented by another which comprises 5-azacytidine and any other active ingredient would infringe the instant claim. Examiner respectfully suggests cancellation is one possible solution. See also claims **31, 57 and 72** wherein the same issue reoccurs.

Applicant's arguments filed August 31, 2012 have been fully considered but they are not persuasive.

Applicant's amendment is noted but has not effectively addressed the issue. Cancellation of the noted claims or submission of additional relevant test data in the form of a declaration filed under 37 C.F.R. §1.132 may effectively address the issue.

In claim **51** beginning at lines 4-6, the term "wherein the composition is for treating ... leukemia" is a method of treating limitation and therefore has no patentable weight in a pharmaceutical composition claim. Deletion is respectfully requested. If said deletion is executed, then claim **51** and claims dependent therefrom would become substantial equivalents to, or duplicates of, claims **1, 3-22 and 72**. Deletion of duplicate claims is also respectfully suggested.

Applicant's arguments with respect to claims **1, 3-55, 57, 59-61 and 66-71** have been considered but are moot in view of the new grounds of rejection. This new ground of rejection was necessitated by applicant's amendments.

The following is a quotation of 35 U.S.C. §103(**a**) which forms the basis for all obviousness rejections set forth in this Office action:

> "A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made."

Claims **1, 3-22, 51-55, 57, 59-61 and 72** are rejected under 35 U.S.C. §103(**a**) as being unpatentable in view of applicant's admissions at page 3, paragraph **[0009]**, wherein applicant has admitted that 5-azacytidine is a compound well known in the art, and in view of the following precedents.

Applicant is requested to note that claiming an unpatentable compound in combination with a carrier does not render the combination patentable if it would be obvious in the prior art to utilize a carrier with the compound: see *In re Lerner* , (CCPA 1971) 438 F2d 1008; 169 USPQ 51; and *In re Rosicky* , (CCPA 1960) 276 F 2d 656, 125 USPQ 341.

Therefore, the instant claimed pharmaceutical compositions comprising 5-azacytidine would have been obvious to one of ordinary skill in the art having the above cited reference before him at the time the invention was made.

Claims **1, 3-23, 31-55, 57, 59-61 and 72** are rejected under 35 U.S.C. §103(**a**) as being unpatentable over **Redkar et al. '046** (PTO-892 ref. **B**: US **2006/0074046**) in view of **Dintaman et al.** (PTO-892 ref. **R**) and further in view of **Sands et al. '263** (PTO-1449 ref. **A09**: US **2004/01652263**).

The instant claims are directed to pharmaceutical compositions comprising 5-azacytidine, an optional penetration enhancer, and various other notoriously well known in the art excipients, and to a method of treating various neoplastic diseases as part of the indefinite genus of "diseases associated with abnormal cell proliferation" (MDS, AML non-small cell lung cancer, ovarian cancer, pancreatic cancer, psoriasis, etc.) therewith.

Redkar et al. '046 discloses at paragraphs **[0003]** and **[0011]**, that 5-azacytidine and salts thereof (paragraph **[0031]**) are active ingredients, that the pharmaceutical compositions thereof are known (paragraphs **[0175]** to **[0182]**), and that said compositions are effective following oral administration in the treatment of neoplastic diseases (Abstract, paragraphs **[0031]** to **[0034]**, and **[0106]**). At paragraph **[0009]** the **'046** reference teaches that both the aqueous solution instability plus the low water solubility of 5-azacytidine has made administration of aqueous solution thereof problematic, difficulties apparently effectively reversed by substitution of acid salts thereof. At paragraph **[0121]** the **'046** reference teaches that strong acid salts of 5-azacytidine have been found to have improved solubility and to be more stable, apparently because of ion pair formation as taught in paragraphs **[0107]** and **[0117]**. And at paragraphs **[0181]** and **[0182]** the **'046** reference teaches the co-administration of 5-azacytidine with numerous generic classes of excipients and carriers, and also with a second active ingredient.

Although **Redkar et al. '046** does disclose many varieties of carriers and excipients as part of 5-azacytidine-containing tablets, this reference does not expressly disclose pharmaceutical compositions comprising a "permeation enhancer" including the vitamin E derivative TPGS, limitations only found in instant claims **7-9 and 33-35**, or the administration thereof to a host in need thereof.

Dintaman et al. discloses the vitamin E derivative TPGS and further discloses its mechanism of action as an inhibitor of P-glycoproteins known to be part of chemotherapy failures due to multidrug resistance. Reversal of this multidrug resistance effect is illustrated

with pharmaceutical compositions comprising a known antibiotic and a known anti-neoplastic agent plus TPGS.

Sands et al. '263 discloses pharmaceutical compositions comprising 5-azacytidine at paragraph **[0029]**, and carriers useful therein at paragraphs **[0057]** to **[0059]** including the carriers specified in instant claim **6**. This reference also discloses at paragraph **[0038]** that the pharmaceutical compositions include the capability to rapidly dissolve in mildly acidic solutions. In addition at paragraph **[0065]** this reference also teaches that the pharmaceutical compositions may also include substances which can "enhance the therapeutic efficacy" of the composition. And finally the **'263** reference teaches at paragraphs **[0056]** -**[0061]** that multiple different excipients may be combined with 5-azacytidine in the manufacture of tablets or capsules, and wherein said tablets may be prepared without an enteric coating (see paragraph **[0037]**), an alternative that appears to be an equivalent to the instant "immediate release" type of solid dosage vehicle claimed herein.

It would have been obvious to a person of ordinary skill in the art at the time the invention was made to add the instant claims excipients or carriers to the pharmaceutical compositions of the **Redkar et al. '046** reference because the **Redkar et al. '046** reference provides teachings which permits or suggests that such excipients are part of the disclosure therein. The **'046** reference also teaches the advantages of increased solubility and stability associated with substitution of strong acid salts of 5-azacytidine for the neutral compound, a teaching which appears to render obvious the instant "immediate release" approach to the oral administration of 5-azacytidine tablets and capsules, a variation which implies the rapid conversion the the active ingredient, 5-azacytidine, into its apparently very stable and very water soluble strong acid salt in the stomach of the host being treated. In addition the disclosures of the **Sands et al. '263** reference provide broad coverage for alternative excipients including the specific excipients claimed herein, including compounds like the compound TPGS disclosed in the **Dintaman et al.** reference.

One having ordinary skill in the art would have been motivated to combine these references because all three references are directed to pharmaceutical compositions and the administration thereof to treat neoplastic diseases, including the treatment of MDS, by the administration of either 5-azacytidine, as disclosed in the two cited U. S. patent publications, or alternative medicinal agents as disclosed by **Dintaman et al.**

Therefore, the instant claimed method of treating " ... diseases associated with abnormal cell proliferation," including neoplastic diseases, by the oral administration of 5-azacytidine as the active ingredient, and optionally with TPGS as a permeation enhancer, in an "immediate release" pharmaceutical composition to a host in need thereof would have been obvious to one of ordinary skill in the art having the above cited reference before him at the time the invention was made.

Applicant's arguments filed August 31, 2012 have been fully considered but they are not persuasive.

Applicant has alleged that the **Redkar '046** reference is limited in its disclosure to decitabine. Examiner respectfully disagrees, and directs applicant's attention to the **'046** reference at paragraphs **[0003]** and **[0011]** wherein 5-azacytidine has been mentioned twice. In addition decitabine is a close relative, being 2'-deoxy-5-azacytidine, so the solubility characteristics of these two compounds and their acid salts would be expected by the ordinary practitioner to be quite similar.

Applicant then alleges in the paragraph bridging pages 13 and 14 that the **Redkar '046** reference has failed to disclose an "immediate release" pharmaceutical composition comprising 5-azacytidine, but this argument avoids the issue of adequately defining in the instant claims what combination(s) of carriers and excipients is/are necessary to formulate applicant's "immediate release" tablets and capsules.

At page 14 applicant has argued that the **Dintaman et al.** reference is inappropriate based on a very brief and error filed analysis of this reference. This portion of applicant's argument is therefore has not been found to be convincing.

Applicant's argument in re the **Sands '263** reference concerning paragraph **[0064]** have been found convincing and the paragraph reference has been change to **[0037]**.

Applicant's assertion of hindsight reconstruction has been reviewed, but has not been found convincing because the fundamental issue presently unaddressed herein is the failure to provide the identities of the formulations required to cause "immediate release" of the claimed pharmaceutical compositions following oral ingestion. The absence of this limitation stated in

a well defined form makes it difficult to either search the subject matter or determine patentability herein.

For these reasons the instant rejection has been found to remain valid and for this reason has been maintained.

No claim is allowed.

Papers related to this application may be submitted to Group 1600 via facsimile transmission (FAX). The transmission of such papers must conform with the notice published in the Official Gazette (1096 OG 30, November 15, 1989). The telephone number to FAX (unofficially) directly to Examiner's computer is 571-273-0651. The telephone number for sending an Official FAX to the PTO is 571-273-8300.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Examiner L. E. Crane whose telephone number is **571-272-0651**. The examiner can normally be reached between 9:30 AM and 5:00 PM, Monday through Friday.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Ms. S. Anna Jiang, can be reached at **571-272-0627**.

Any inquiry of a general nature or relating to the status of this application should be directed to the Group 1600 receptionist whose telephone number is **571-272-1600**.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status Information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see < http://pair-direct.uspto.gov >. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at **866-217-9197** (toll-free).

sd

LECrane:lec
08/26/2013

/Lawrence E. Crane/

Primary Examiner, Art Unit 1623

L. E. Crane
Primary Patent Examiner
Technology Center 1600

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	12466213	ETTER ET AL.
	Examiner	Art Unit
	LAWRENCE E CRANE	1623

CPC- SEARCHED		
Symbol	Date	Examiner

CPC COMBINATION SETS - SEARCHED		
Symbol	Date	Examiner

US CLASSIFICATION SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES		
Search Notes	Date	Examiner
Classification for restriction purposes - 514/43; 535/28.3	7/25/2011	LEC
File CAPLUS search - see search for strategy	7/25/2011	LEC
Inventor name search - (Etter)	7/25/2011	LEC

INTERFERENCE SEARCH			
US Class/ CPC Symbol	US Subclass / CPC Group	Date	Examiner

	/LAWRENCE E CRANE/ Primary Examiner.Art Unit 1623

LIST OF REFERENCES CITED BY APPLICANT (Use several sheets if necessary)		
	Application Number	**12/466,213**
	Filing Date	May 14, 2009
	First Named Inventor	Jeffrey B. Etter
	Art Unit	1623
	Examiner Name	Lawrence E. Crane
	Attorney Docket No.	9516-847-999

U.S. PATENT DOCUMENTS

*Examiner Initials	Cite No.	Document Number – Kind Code	Publication Date mm/dd/yyyy	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
AA3	A47	US 8,211,862	07/03/2012	Ionesco *et al.*	

FOREIGN PATENT DOCUMENTS

*Examiner Initials	Cite No.	Foreign Patent Document Country Code, Number, Kind Code (if known)	Publication Date mm/dd/yyyy	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	T
AL3	B10	WO 2004/082619	09/30/2004	Pharmion Corporation		
AM3	B11	WO 2006/034154	03/30/2006	Supergen, Inc.		
AN3	B12	WO 2008/088779	07/24/2008	Ivax Pharmaceuticals S.R.O. et al.		

NON PATENT LITERATURE DOCUMENTS

*Examiner Initials	Cite No.	Include name of the author (in CAPITAL LETTERS), (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T
AR3	C71	Notice of Allowability in U.S. Patent Application No. 12/729,116, mailed May 4, 2012.	

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /L.C./

SDI-136025v1

EXAMINER SIGNATURE	/Lawrence Crane/	DATE CONSIDERED	08/24/2013

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	12466213	ETTER ET AL.
	Examiner	Art Unit
	LAWRENCE E CRANE	1623

✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
=	Allowed	÷	Restricted	I	Interference	O	Objected

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

CLAIM		DATE								
Final	Original	07/25/2011	02/24/2012	08/25/2013						
	1	✓	✓	✓						
	2	✓	-	-						
	3	✓	✓	✓						
	4	✓	✓	✓						
	5	✓	✓	✓						
	6	✓	✓	✓						
	7	✓	✓	✓						
	8	✓	✓	✓						
	9	✓	✓	✓						
	10	✓	✓	✓						
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	28	✓	✓	-						
	29	✓	✓	-						
	30	✓	✓	-						
	31	✓	✓	✓						
	32	✓	✓	✓						
	33	✓	✓	✓						
	34	✓	✓	✓						
	35	✓	✓	✓						
	36	✓	✓	✓						

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	12466213	ETTER ET AL.
	Examiner	Art Unit
	LAWRENCE E CRANE	1623

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CLAIM		DATE								
Final	Original	07/25/2011	02/24/2012	08/25/2013						
	37	✓	✓	✓						
	38	✓	✓	✓						
	39	✓	✓	✓						
	40	✓	✓	✓						
	41	✓	✓	✓						
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	63	✓	-	-						
	64	✓	-	-						
	65	✓	-	-						
	66		✓	-						
	67		N	-						
	68		✓	-						
	69		N	-						
	70		✓	-						
	71		N	-						
	72			✓						

LIST OF REFERENCES CITED BY APPLICANT (Use several sheets if necessary)		
	Application Number	12/466,213
	Filing Date	May 14, 2009
	First Named Inventor	Jeffrey B. ETTER
	Art Unit	1623
	Examiner Name	CRANE, Lawrence E.
	Attorney Docket No.	9516-847-999
	CAM No.	501872-999847

U.S. PATENT and PUBLICATION DOCUMENTS

*Examiner Initials		Document Number	Date yyyy-mm-dd	Name of Patentee or Applicant of Cited Document	
AA4	A48	US 2012/0196823	2012-08-02	Tutino et al.	
AB4	A49	US 2013/0109644	2013-05-02	MacBeth et al.	

FOREIGN PATENT DOCUMENTS

*Examiner Initials		Foreign Patent Document Country Code, Number, Kind Code (if known)	Date yyyy-mm-dd	Name of Patentee or Applicant of Cited Document		T
AL4	B13	WO 2004/082822	2004-09-30	Pharmion Corporation		
AM4	B14	WO 2006/089290	2006-08-24	American Bioscience Inc.		
AN4	B15	WO 2009/139888	2009-11-19	Celgene Corporation		
AO4	B16	WO 2010/059969	2010-05-27	Genentech Inc.		
AP4	B17	WO 2012/135405	2012-10-04	Pharmion LLC		
AQ4	B18	WO 2013/022872	2013-02-14	Celgene Corporation		

NON PATENT LITERATURE DOCUMENTS

*Examiner Initials		Include name of the author (in CAPITAL LETTERS), (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T
	C72 AR4	ANONYMOUS, "A Phase I/II Clinical Trial of Vidaza with Abraxane in the Treatment of Patients with Advanced or Metastatic Solid Tumors and Breast Cancer," ClinicalTrials.gov archive, pages 1-3, retrieved from the Internet: http://clinicaltrials.gov/archive/NCT00748553/2011_08_05, on January 31, 2013	
	C73 AS4	ANONYMOUS, "Oral Azacitidine as a Single Agent and in Combination with Carboplatin or Abraxane in Subjects with Relapsed or Refractory Solid Tumors," ClinicalTrials.gov archive, pages 1-4, retrieved from the Internet: http://clinicaltrials.gov/archive/NCT01478685/2011_11_22, on January 31, 2013	
	C74 AT4	BAST et al., "A Phase IIa Study of a Sequential Regimen Using Azacitidine to Reverse Platinum Resistance to Carboplatin in Patients with Platinum Resistant or Refractory Epithelial Ovarian Cancer," Journal of Clinical Oncology, 26, Abstract 3500, (2008)	
	C75 AU4	COWAN et al., "Will DNA Methylation Inhibitors Work in Solid Tumors? A Review of the Clinical Experience with Azacitidine and Decitabine in Solid Tumors," Epigenomics, Future Medicine Ltd, United Kingdom, 2(1):71-86 (2010)	
	C76 AV4	GLASER, "HDAC Inhibitors: Clinical Update and Mechanism-Based Potential," Biochem. Pharm., 74:659–71 (2007)	

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /L.C./

SDI-162357v1

EXAMINER	/Lawrence Crane/	DATE CONSIDERED	08/24/2013

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

LIST OF REFERENCES CITED BY APPLICANT (Use several sheets if necessary)		
	Application Number	12/466,213
	Filing Date	May 14, 2009
	First Named Inventor	Jeffrey B. ETTER
	Art Unit	1623
	Examiner Name	CRANE, Lawrence E.
	Attorney Docket No.	9516-847-999
	CAM No.	501872-999847

NON PATENT LITERATURE DOCUMENTS

*Examiner Initials		Include name of the author (in CAPITAL LETTERS), (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T
	C77 AW4	HOWELL et al., "Demethylating Agents in the Treatment of Cancer," Pharmaceuticals, 3(7):2022–44 (2010)	
	C78 AX4	JUERGENS et al., "Interim Analysis of a Phase II Trial of 5-Azacitidine (5AC) and Entinostat (SNDX-275) in Relapsed Advanced Lung Cancer (NSCLC)," Journal of Clinical Oncology, 27(15S):8055 (2009)	
	C79 AY4	MOMPARLER, "Epigenetic Therapy of Cancer with 5-Aza-2'-Deoxycitidine (Decitabine)," Seminars in Oncology, 32(5):443–51 (2005)	
	C80 AZ4	STATHIS et al., "Phase I Study of Decitabine in Combination with Vorinostat in Patients with Advanced Solid Tumors and Non-Hodgkin's Lymphomas," Clinical Cancer Research, 17(6):1582–90 (2011)	
BR4	C81	Vidaza™ Label (azacitidine for injectable suspension), Version: 5-18-04.	
BS4	C82	Vidaza™ (azacitidine for injection) Prescribing Information, dated January 2012.	

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /L.C./

SDI-162357v1

EXAMINER	/Lawrence Crane/	**DATE CONSIDERED**	08/24/2013

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/466,213	05/14/2009	Jeffrey B. Etter	9516-847-999/501872-847	5370

84802 7590 02/03/2014

JONES DAY for Celgene Corporation
222 E. 41ST. STREET
NEW YORK, NY 10017

EXAMINER	
CRANE, LAWRENCE E	
ART UNIT	**PAPER NUMBER**
1673	

MAIL DATE	DELIVERY MODE
02/03/2014	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Applicant-Initiated Interview Summary	Application No.	Applicant(s)	
	12/466,213	ETTER ET AL.	
	Examiner	Art Unit	
	Lawrence E. Crane	1673	

All participants (applicant, applicant's representative, PTO personnel):

(1) *Lawrence E. Crane (USPTO)*.

(2) *Robert Chang & Yeah-Sil Moon (Jones Day)*.

(3) *C. L. Beach (Celgene)*.

(4) *Donna Robertson-Chow (Celgene)*.

Date of Interview: *29 January 2014*.

Type: ☒ Telephonic ☐ Video Conference
☐ Personal [copy given to: ☐ applicant ☐ applicant's representative]

Exhibit shown or demonstration conducted: ☒ Yes ☐ No.
If Yes, brief description: *Applicant provided additional references in advance by e-mail*.

Issues Discussed ☐101 ☒112 ☐102 ☒103 ☒Others
(For each of the checked box(es) above, please describe below the issue and detailed description of the discussion)

Claim(s) discussed: *1,3-23,31-55,57,59-61 and 72*.

Identification of prior art discussed: *Prior art supplied by applicant by e-mail but not yet of record*.

Substance of Interview
(For each issue discussed, provide a detailed description and indicate if agreement was reached. Some topics may include: identification or clarification of a reference or a portion thereof, claim interpretation, proposed amendments, arguments of any applied references etc...)

An extensive presentation (> 1 hr) including both suggestions and questions by the examiner was conducted. Applicant was advised that the presentation was useful but that the points made needed to be made of record in the form of one or more declarations filed under 37 CFR 1.132 in addition to the normal response to be filed after a first action. Examiner requested and applicant agreed to supply all additional references relevant to the discussion and to make same of record on a PTO-1449. Examiner did provided limited advise on how the claims of record might be amended, noting that negative limitations are not preferred in view of 112, second paragraph, and speculating that only method of treating claims may be allowable. However, because examiner has not yet seen applicant's anticipated submissions, no agreement could be reached concerning allowability.

Applicant recordation instructions: The formal written reply to the last Office action must include the substance of the interview. (See MPEP section 713.04). If a reply to the last Office action has already been filed, applicant is given a non-extendable period of the longer of one month or thirty days from this interview date, or the mailing date of this interview summary form, whichever is later, to file a statement of the substance of the interview

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

☐ Attachment

/Lawrence E. Crane/ Primary Examiner, Art Unit 1673	

Summary of Record of Interview Requirements

Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record
A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews
Paragraph (b)

In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.
All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

———

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiner's responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicant's correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:
- Application Number (Series Code and Serial Number)
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (telephonic, video-conference, or personal)
- Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
- An indication whether or not an exhibit was shown or a demonstration conducted
- An identification of the specific prior art discussed
- An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
- The signature of the examiner who conducted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind the applicant of his or her obligation to record the substance of the interview of each case. It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview.

A complete and proper recordation of the substance of any interview should include at least the following applicable items:
1) A brief description of the nature of any exhibit shown or any demonstration conducted,
2) an identification of the claims discussed,
3) an identification of the specific prior art discussed,
4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the Examiner,
5) a brief identification of the general thrust of the principal arguments presented to the examiner,
   (The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he or she feels were or might be persuasive to the examiner.)
6) a general indication of any other pertinent matters discussed, and
7) if appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the examiner.

Examiners are expected to carefully review the applicant's record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extendable one month time period to correct the record.

Examiner to Check for Accuracy

If the claims are allowable for other reasons of record, the examiner should send a letter setting forth the examiner's version of the statement attributed to him or her. If the record is complete and accurate, the examiner should place the indication, "Interview Record OK" on the paper recording the substance of the interview along with the date and the examiner's initials.

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (01-10)
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	Filing Date	2009-05-14
	First Named Inventor	Jeffrey B. Etter
	Art Unit	1673
	Examiner Name	Lawrence E. Crane
	Attorney Docket Number	9516-847-999

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	1	GARCIA-MANERO et al., "Safety and efficacy of oral azacitidine (CC-486) administered in extended treatment schedules to patients with lower-risk myelodysplastic syndromes," Blood, 120:Abstract 424 (2012).	☐
	2	GARCIA-MANERO et al., "Phase I study; of oral azacitidine in myelodysplastic syndromes, chronic myelomonocytic leukemia, and acute myeloid leukemia," J. Clin. Oncol., 29:2521-2527 (2011).	☐
	3	Vidaza™ (azacitidine for injection) Prescribing Information, dated December 2012.	☐

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Blood (ASH Annual Meeting Abstracts) 2012 120: Abstract 424

Oral Sessions

633. Myelodysplastic Syndromes: Updates on Therapy and Prognosis

Safety and Efficacy of Oral Azacitidine (CC-486) Administered in Extended Treatment Schedules to Patients with Lower-Risk Myelodysplastic Syndromes

Guillermo Garcia-Manero, MD[1], Steven D. Gore, MD[2], Suman Kambhampati, MD[*,3], Bart L Scott, MD, MS[4], Ayalew Tefferi, MD[5], Christopher R Cogle, MD[6], William Edenfield, MD[*,7], Joel Hetzer, PhD[*,8], Keshava Kumar, PhD[*,8] and Barry S. Skikne, MD[9]

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Abstract 424

Background: Azacitidine for injection has been shown to prolong overall survival in patients (pts) with higher-risk myelodysplastic syndromes (MDS) compared with conventional care regimens (CCR) (*Lancet Oncol*, 2009). An oral formulation of azacitidine (CC-486) is in development. Oral azacitidine may maximize convenience, eliminate injection-site reactions, and if administered in extended dosing schedules, may enhance and prolong the therapeutic effects of azacitidine. Oral azacitidine administered once-daily (QD) for 7 days (d) of repeated 28d cycles has been shown to be bioavailable, biologically and clinically active, and well-tolerated in pts with MDS and acute myeloid leukemia (Garcia-Manero, *J Clin Oncol*, 2011). Preliminary evidence suggests that

extending oral azacitidine dosing to 14d or 21d of the 28d cycle may enhance pharmacodynamic and epigenetic activity (Laille, *Leuk Res*, 2011).

Purpose: To evaluate hematologic response and safety associated with extended dosing regimens of oral azacitidine in pts with lower-risk MDS.

Methods: This ongoing, multicenter, phase 1 study, enrolled pts with lower-risk (IPSS Low or INT-1) MDS who were RBC transfusion dependent (TD) and/or thrombocytopenic (average platelet count ≤50,000 within 56d prior to the first dose) at baseline. Pts were sequentially assigned to receive oral azacitidine 300mg QD for either 14d or 21d of repeated 28d cycles. Hematologic assessments were made every 2 weeks. Hematologic response was assessed using IWG 2006 criteria (Cheson, *Blood*, 2006). Adverse events (AEs) were graded using NCI-CTCAE version 3.0.

Results: At data cut-off (May 18, 2012), 53 pts with lower-risk MDS had enrolled (300mg oral azacitidine QDx14d, n=26; QDx21d, n=27). Demographic and disease characteristics at baseline were similar in the 14d and 21d treatment cohorts (**Table 1**). Median (range) hematology counts at baseline were Hgb 8.7 g/L (6.0–13.0), ANC $1.6x10^9$/L (0–30.3), and platelets $56.0x10^9$/L (6.0–564.0). At study entry, 40% of pts had received no prior MDS treatment (except transfusions), 45% had received erythropoiesis-stimulating agents, and 15% had received WBC growth factors. The number of oral azacitidine treatment cycles received ranged from 1 to 12 (median numbers of oral azacitidine cycles were 6 in the QDx14d and 4 in the QDx21d cohorts). Four pts in the 21d cohort and 1 pt in the 14d cohort received reduced oral azacitidine doses (200mg QD). Overall, 10 pts discontinued the study, including 6 pts (3 pts in each cohort) who discontinued due to AEs that may have been treatment-related (gastrointestinal [n=2] or intracranial [n=1] hemorrhage, febrile neutropenia [n=1], pneumonia [n=1], thrombocytopenia [n=1]). Overall response rates (ORR), which included complete (CR) and partial remission (PR), any hematologic improvement (HI), and transfusion independence (TI), ranged from 38.5% in the QDx14d cohort to 29.6% in the QDx21d cohort, and RBC TI was achieved by 47% and 33%, respectively, of pts who were RBC TD at baseline (**Table 2**). For pts who received at least 4 cycles of oral azacitidine (14d, n=19; 21d, n=14), ORR was 47.4% in the 14d and 50.0% in the 21d cohorts, and RBC TI rates in RBC TD pts (n=16) were 67% in the 14d and 57% in the 21d cohorts. The most frequent (≥5%) grade 3/4 hematologic AEs in the QDx14d cohort were anemia (11.5%), thrombocytopenia (11.5%), and neutropenia (7.7%); and in the QDx21d cohort were neutropenia (14.8%), anemia (7.4%), and febrile neutropenia (7.4%). Most frequent grade 3/4 non-hematologic AEs were gastrointestinal, including vomiting (7.7%) in the QDx14d cohort, and diarrhea (11.1%) and vomiting (7.4%) in the QDx21d cohort.

Conclusions: Oral azacitidine 300mg QD administered in extended dosing schedules of 14d or 21d of repeated 28d cycles was effective and well-tolerated in these pts with lower-risk MDS. Beside hematologic AEs, the most frequently observed AEs with oral azacitidine were gastrointestinal and were manageable. Efficacy and safety outcomes with 300mg QD oral azacitidine were generally comparable between the 14d and 21d extended dosing regimens. Based on these data, oral azacitidine administered once-daily

in extended dosing schedules is active and well-tolerated and warrants further investigation in randomized, controlled trials.

Table 1. Patient Demographic and Disesse Characteristics at Baseline

Table 1. Patient Demographic and Disease Characteristics at Baseline

Characteristic	14-day QD (N=39)	21-day QD (n=27)
Age, years Median (range)	73.0 (70 – 77)	70.0 (62 – 76)
RBC transfusion dependent,* n (%)	15 (38)	15 (56)
MDS WHO classification, n (%) RA/RARS RCMD/RCMD-RS RAEB-1 RAEB-2 MDS-U Del (5q)	 8 (21) 9 (35) 4 (15) 0 3 (8) 3 (8)	 9 (33) 7 (26) 4 (15) 1 (4) 3 (11) 1 (4)
IPSS risk classification, n (%) Low Intermediate-1	 6 (23) 20 (77)	 9 (33) 18 (67)
Cytogenetics, n (%) Normal/diploid ≥1 Abnormality Indeterminate	 11 (42) 11 (42) 4 (15)	 15 (56) 9 (33) 3 (11)
Prior treatment, n (%) Erythropoiesis-stimulating agents WBC growth factors Other None†	 14 (54) 4 (15) 7 (27) 9 (35)	 10 (37) 4 (15) 5 (19) 12 (44)

*RBC transfusion dependence at baseline was defined as receipt of ≥8 units of packed RBC within 56 days of the first dose of oral azacitidine.
†Other than transfusions.

Table 2. Hematologic Response and Transfusion Independence with Oral Azacitidine 300mg QD in Extended Dosing Schedules (124 and 21d) in Patients with Lower-risk MDS

Table 2. Hematologic Response and Transfusion Independence with Oral Azacitidine 300mg QD in Extended Dosing Schedules (14d and 21d) in Patients with Lower-risk MDS

Parameter	Treatment Schedule (Independent IVRS Committee, IEC)		
	14 days QD (n=26)	21 days QD (n=77)	Total (N=103)
Overall Response*† (CR, PR, any HI, TI)	10/26 (38.5)	8/77 (10.4)	18/103 (34.0)
Any HI	6/26 (23.1)	7/77 (10.4)	13/103 (12.6)
HI – E	4/23 (17.4)	6/75 (14.0)	10/98 (10.2)
HI – P	4/37 (10.8)	2/35 (5.7)	6/72 (8.3)
HI – N	2/39 (5.1)	0/6	2/45 (4.4)
Marrow CR (mCR)	0/7	3/5 (60.0)	3/12 (25.0)
RBC transfusion independence (TI)‡ Sustained for 56 days Sustained for 84 days	7/15 (46.7) 3/15 (20.0)	5/15 (33.3) 4/15 (26.7)	12/30 (40.0) 7/30 (23.3)

*IWG 2006 criteria.
†Patients are counted only once for overall response, but may be counted more than once in individual response categories.
‡To be evaluated for TI, pts must have been RBC transfusion dependent (TD) at baseline and been on study at least 56 days.
RBC TD at baseline was defined as receipt of ≥4 units of packed RBC within 56 days of the first dose of oral azacitidine.

Disclosures: Garcia-Manero: *Celgene:* Research Funding, Speakers Bureau. **Gore:** *Celgene Corporation:* Consultancy, Research Funding. **Scott:** *Celgene Corporation:* Honoraria, Research Funding, Speakers Bureau. **Hetzer:** *Celgene Corporation:* Employment, Equity Ownership. **Kumar:** *Celgene Corporation:* Employment, Equity Ownership. **Skikne:** *Celgene:* Employment, Equity Ownership.

Footnotes

* Asterisk with author names denotes non-ASH members.

VOLUME 29 · NUMBER 18 · JUNE 20 2011

JOURNAL OF CLINICAL ONCOLOGY

ORIGINAL REPORT

Phase I Study of Oral Azacitidine in Myelodysplastic Syndromes, Chronic Myelomonocytic Leukemia, and Acute Myeloid Leukemia

Guillermo Garcia-Manero, Steven D. Gore, Christopher Cogle, Renee Ward, Tao Shi, Kyle J. MacBeth, Eric Laille, Heidi Giordano, Sarah Sakoian, Elias Jabbour, Hagop Kantarjian, and Barry Skikne

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Submitted December 21, 2010; accepted March 22, 2011; published online ahead of print at www.jco.org on May 16, 2011.

Supported by Celgene; editorial support in the preparation of this manuscript from Excerpta Medica, sponsored by Celgene.

Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

Clinical Trials repository link available on JCO.org.

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0732-183X/11/2918-2521/$20.00

DOI: 10.1200/JCO.2010.34.4226

ABSTRACT

Purpose

To determine the maximum-tolerated dose (MTD), safety, pharmacokinetic and pharmacodynamic profiles, and clinical activity of an oral formulation of azacitidine in patients with myelodysplastic syndromes (MDSs), chronic myelomonocytic leukemia (CMML), or acute myeloid leukemia (AML).

Patients and Methods

Patients received 1 cycle of subcutaneous (SC) azacitidine (75 mg/m^2) on the first 7 days of cycle 1, followed by oral azacitidine daily (120 to 600 mg) on the first 7 days of each additional 28-day cycle. Pharmacokinetic and pharmacodynamic profiles were evaluated during cycles 1 and 2. Adverse events and hematologic responses were recorded. Cross-over to SC azacitidine was permitted for nonresponders who received ≥ 6 cycles of oral azacitidine.

Results

Overall, 41 patients received SC and oral azacitidine (MDSs, n = 29; CMML, n = 4; AML, n = 8). Dose-limiting toxicity (grade 3/4 diarrhea) occurred at the 600-mg dose and MTD was 480 mg. Most common grade 3/4 adverse events were diarrhea (12.2%), nausea (7.3%), vomiting (7.3%), febrile neutropenia (19.5%), and fatigue (9.8%). Azacitidine exposure increased with escalating oral doses. Mean relative oral bioavailability ranged from 6.3% to 20%. Oral and SC azacitidine decreased DNA methylation in blood, with maximum effect at day 15 of each cycle. Hematologic responses occurred in patients with MDSs and CMML. Overall response rate (ie, complete remission, hematologic improvement, or RBC or platelet transfusion independence) was 35% in previously treated patients and 73% in previously untreated patients.

Conclusion

Oral azacitidine was bioavailable and demonstrated biologic and clinical activity in patients with MDSs and CMML.

J Clin Oncol 29:2521-2527. © 2011 by American Society of Clinical Oncology

INTRODUCTION

Azacitidine is a cytidine nucleoside analog with a mechanism of action that involves incorporation into DNA and RNA.[1,2] Data suggest that patients must be exposed to azacitidine over several treatment cycles for optimal therapeutic effect.[3] The requirement for chronic exposure can be explained by drug pharmacokinetics, as azacitidine has a short plasma half-life, and by mechanism of action, as induction of DNA hypomethylation through incorporation into DNA is cell-cycle dependent (S-phase restricted) and DNA remethylation is observed by the end of each treatment cycle.[4]

A treatment regimen facilitating chronic administration may help achieve optimal efficacy outcomes. An oral azacitidine formulation would improve convenience of administration and expand the possibilities of exploring novel maintenance schedules, targeting different malignancies, and testing multiple combinations. A phase 0 trial demonstrated that a single oral azacitidine dose resulted in detectable levels in the blood.[5]

This phase I study sought to identify the maximum-tolerated dose (MTD), dose-limiting toxicities (DLTs), safety, pharmacokinetic and pharmacodynamic profiles, and clinical activity of oral azacitidine in patients with myelodysplastic syndromes (MDSs), chronic myelomonocytic leukemia (CMML), or acute myeloid leukemia (AML).

PATIENTS AND METHODS

The trial was approved by the relevant institutional review boards and ethics committees. All patients gave written informed consent.

Patients

Eligible patients were ≥ 18 years, had an Eastern Cooperative Oncology Group performance status score of 0 to 2, and a diagnosis of MDSs, CMML, or AML according to WHO classification.[6,7] For patients with AML, eligibility was limited to those for whom standard curative measures did not exist or were no longer effective. Exclusion criteria included a diagnosis of acute promyelocytic leukemia, previous treatment with hypomethylating agents within 4 weeks before cycle 1, and anticancer therapy within 21 days before the first dose of study drug, or less than full recovery from any significant toxic effects of prior treatments.

Table 1. Baseline Patient Characteristics (N = 41)

Parameter	No. of Patients	%
Median age, years	70	
Range	31-91	
Sex		
Male	32	78
Female	9	22
MDSs (WHO classification)	29	71
RA/RARS/RCMD	11	27
RAEB-1	12	29
RAEB-2	5	12
MDSs-U	1	2
CMML	4	10
AML	8	20
De novo	4	10
Transformed from MDSs	4	10
IPSS (MDSs patients)*		
Low risk	2	7
Intermediate 1 risk	12	41
Intermediate 2 risk	13	45
High risk	1	3
Not available†	1	3
Hematology		
Median hemoglobin, g/dL	9.3	
Range	6.9-15.1	
Median white blood cell count $\times 10^9$/L	2.4	
Range	0.4-30.2	
Median absolute neutrophil count $\times 10^9$/L	0.8	
Range	0.0-21.7	
Median platelet count $\times 10^9$/L	54.0	
Range	3.0-262.0	
Cytogenetics‡		
Normal chromosomal karyotype	17	49
1 chromosomal abnormality	9	26
2 chromosomal abnormalities	3	9
≥ 3 chromosomal abnormalities	6	17
Prior treatment with hypomethylating agent	16	39
MDSs	13	32
CMML	0	0
AML	3	7

Abbreviations: AML, acute myeloid leukemia; CMML, chronic myelomonocytic leukemia; IPSS, International Prognostic Scoring System; MDSs, myelodysplastic syndromes; MDSs-U, MDSs unclassified; RA, refractory anemia; RAEB, RA with excess blasts; RARS, RA with ringed sideroblasts; RCMD, refractory cytopenias with multilineage dysplasia.

*IPSS score[11] was available for 28 patients with MDSs.

†Patient had a bone marrow transplantation and therefore IPSS risk was not considered applicable.

‡Cytogenetic data were available for 35 patients.

Study Design and Therapy

This open-label, phase 1, dose-escalation trial was performed in four participating institutions and evaluated multiple cycles of oral azacitidine administered daily for the first 7 days of a 28-day cycle. The objectives were to determine the MTD, DLTs, and the safety profile of oral azacitidine. Pharmacokinetic and pharmacodynamic profiles of oral and subcutaneous (SC) azacitidine, administered on the same 7-day schedule, were also compared. A secondary objective was to assess the clinical activity of oral azacitidine.

During cycle 1, patients received azacitidine 75 mg/m^2 daily SC for 7 days of a 28-day cycle. During cycle 2 and beyond, patients received oral azacitidine under fasting conditions (ie, no food for 2 hours before and after dosing). The dose of oral azacitidine was escalated following a standard phase I 3 + 3 design. The starting dose was 120 mg and doses were escalated in 60 mg increments up to a dose of 360 mg, followed by 120 mg increments until the MTD was reached. Intrapatient dose escalation was permitted if the dose level to which the patient was escalated was associated with a DLT rate of ≤ 33%. Treatment continued until disease progression, lack of activity, unacceptable toxicity, or patient preference.

The MTD was defined as the highest dose at which no more than 33% of patients experienced a DLT. DLT was defined as: grade ≥ 3 nausea, diarrhea, or vomiting despite adequate/maximal medical intervention; grade ≥ 3 clinically significant nonhematologic toxicity unrelated to underlying disease or intercurrent illness; failure to recover to an absolute neutrophil count (ANC) of higher than 500/μL and/or platelet count of higher than 25,000/μL with hypocellular bone marrow (< 5%) 42 days after starting oral azacitidine (patients with a baseline ANC of ≤ 500/μL and/or platelet count of ≤ 25,000/μL were not evaluable for neutrophil or platelet toxicity); any treatment-related effect resulting in missing ≥ 3 oral azacitidine doses in the 7-day treatment period; or any treatment-related nonhematologic toxicity delaying initiation of the second oral azacitidine cycle by longer than 14 days. Only DLTs that occurred during the first oral azacitidine cycle were considered in determining the MTD. Adverse events were graded using the National Cancer Institute Common Toxicity Criteria for Adverse Events version 3.0.

Table 2. Incidence of Adverse Events According to Severity in ≥ 20% of Patients Treated With Oral Azacitidine (n = 41)

System Organ Class Preferred Term (MedDRA 10.1)	CTCAE Grade 1		2		3		4		Total	
	No.	%	No.	%	No.	%	No.	%	No.	%
Diarrhea	10	24.4	12	29.3	4	9.8	1	2.4	27	65.9
Nausea	8	19.5	10	24.4	3	7.3	0		21	51.2
Constipation	9	22.0	7	17.1	0		0		16	39.0
Vomiting	4	9.8	6	14.6	3	7.3	0		13	31.7
Abdominal pain	6	14.6	4	9.8	0		0		10	24.4
Headache	7	17.1	5	12.2	1	2.4	0		13	31.7
Fatigue	6	14.6	2	4.9	4	9.8	0		12	29.3
Peripheral edema	11	26.8	1	2.4	0		0		12	29.3
Fever	6	14.6	2	4.9	2	4.9	0		10	24.4
Cough	7	17.1	1	2.4	2	4.9	0		10	24.4
Contusion	9	22.0	0		0		0		9	22.0
Dizziness	5	12.2	3	7.3	0		0		8	19.5
Febrile neutropenia	0		0		8	19.5	0		8	19.5

NOTE. This Table includes all adverse events which started during any dosing cycle at which oral azacitidine was administered. Percentages are based on the number of patients who received at least one dose of oral azacitidine. Multiple reports of the same preferred term from a patient are counted only once, using the maximum CTCAE grade.

Abbreviations: CTCAE, National Cancer Institute Common Toxicity Criteria for Adverse Events; MedDRA, Medical Dictionary for Regulatory Activities.

Pharmacokinetic Analysis

Plasma and urine pharmacokinetic evaluation of azacitidine was performed on days 1 and 7 in cycles 1 and 2. Samples were collected up to 8 hours after administration and analyzed using a validated high-performance liquid chromatography/tandem mass spectrometric method. Parameters calculated using noncompartmental method, included maximum observed plasma concentration (C_{max}), time of maximum observed plasma concentration (T_{max}), area under the plasma concentration–time curve from zero to infinity (AUC_{inf}), apparent total clearance (CL/F), relative oral bioavailability (F), and apparent volume of distribution (Vd/F).

Pharmacodynamic Analysis

DNA methylation levels were measured to determine DNA hypomethylating activity of azacitidine when administered SC or orally. Whole blood was collected at baseline and before drug administration on days 3, 8, 15, and 22 of cycle 1, and days 1, 3, 8, 15, 22, and 28 of cycle 2. Genomic DNA was purified from each whole blood sample using the PAXgene Blood DNA System (Qiagen; Valencia, CA). DNA methylation was analyzed using the Infinium HumanMethylation27 BeadArray (Illumina; San Diego, CA). In cycle 1, DNA methylation data were generated from blood samples of 15 patients. For 10 of these patients, data were also generated in cycle 2. A methylation ratio, or beta value, for each locus per sample was calculated as methylated signal/(methylated + unmethylated signal). Those with detection $P \leq .05$ were considered high-quality measures. Samples with more than 25,200 high-quality beta values and 26,304 autosomal loci with high-quality beta values in at least half of the samples were used for analyses. The low-quality beta values were reimputed using the pamr.knnimpute function from the R package pamr.[8] Wilcoxon signed-rank tests were performed to identify loci with significant methylation differences at each post-treatment time point versus baseline; $P < .01$ was considered statistically significant. All statistical analyses were carried out in R (R Foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org).

Clinical Activity

Data for clinical activity were evaluated using International Working Group (IWG) 2006 criteria, with modifications as described below, for patients with MDSs or CMML[9] and IWG 2003 criteria for patients with AML.[10] Complete remission (CR), hematologic improvement (HI), and RBC and platelet transfusion independence (TI) were evaluated for patients with MDSs or CMML. Bone marrow CR (mCR) was also evaluated but not included in the overall response rate. RBC transfusion dependence at baseline was defined as ≥ 4 RBC units in the 56 days before cycle 1. Platelet transfusion dependence

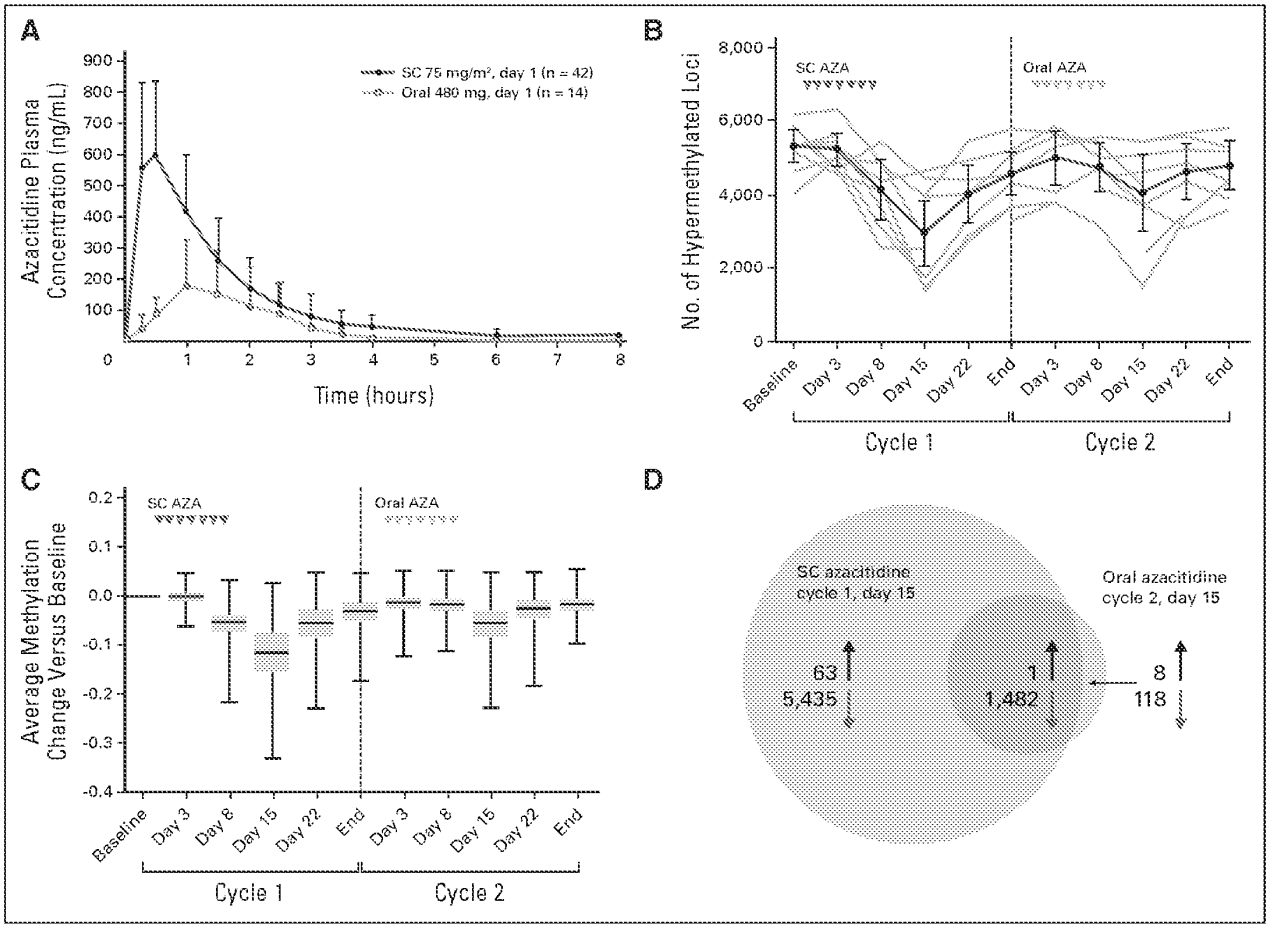

Fig 1. (A) Mean azacitidine (AZA) plasma concentration versus time profiles following single subcutaneous (SC) or oral administration (linear scale). (B) Pharmacodynamics as measured by plotting the numbers of highly methylated loci (beta ≥ 0.7; ± 95% CI) for 10 patients with DNA methylation data in cycles 1 and 2 (gold lines represent individual patients, blue line represents the average). (C) Change in methylation level during treatment with SC or oral AZA for 5,232 loci highly methylated at baseline (blue box represents the 25th to 75th percentile, horizontal band represents the median, vertical line with bars represents minimum and maximum values). (D) Number of significantly differentially methylated loci on day 15 of cycle 1 (SC azacitidine) and on day 15 of cycle 2 (oral azacitidine). Upward arrows denote hypermethylated loci and downward arrows denote hypomethylated loci.

at baseline was defined as ≥ 2 platelet transfusions in the 56 days before cycle 1 (modification to IWG 2006 criteria). RBC and platelet TI were defined as no transfusions in any 56 consecutive-day period on treatment. Patients who achieved ≥ 50% reduction in platelet transfusion requirement, but not platelet TI, in any 56 consecutive-day period on treatment were counted as having achieved HI platelet (HI-P; modification to IWG 2006 criteria). Patients RBC transfusion dependent at baseline achieving a ≥ 50% reduction in RBC transfusion requirement in any 56-consecutive day period and patients not RBC transfusion dependent at baseline, but who achieved a 1.5 g/dL increase in hemoglobin in any 56-consecutive day period on treatment were considered to have achieved HI erythroid (HI-E; modification to IWG 2006 criteria). All patients who received ≥ 1 cycle of oral azacitidine were included in the response analysis. The cutoff date for data in this article was August 19, 2010.

RESULTS

Patient Characteristics

Forty-five patients were treated on a 7-day once-daily schedule. Four patients received the first cycle of SC azacitidine only; three discontinued due to progressive disease (including one death), and one withdrew consent. Baseline characteristics for the remaining 41 patients who received oral azacitidine are presented in Table 1.[11] Cytogenetic data were available at baseline for 35 of 41 patients treated with oral azacitidine; nearly half of the patients had normal karyotype, approximately 25% had a single abnormality, and nearly 20% had a complex karyotype (≥ 3 chromosomal abnormalities). Overall, 16 (39%) of 41 patients had received prior hypomethylating therapy.

Dose Escalation of Oral Azacitidine

No DLTs were observed at dose levels up to 480 mg. DLT was observed at the 600 mg dose, with two (66.7%) of three patients experiencing severe diarrhea, despite adequate medical intervention (grade 3 in one patient and grade 4 in the other). Per protocol, the MTD was exceeded and the previous dose level of 480 mg was determined to be the MTD.

Safety Profile

Table 2 shows the incidence of AEs (any grade) that occurred in ≥ 20% of patients treated with oral azacitidine. The most frequently observed AEs were gastrointestinal disorders, headache, fatigue, and peripheral edema. Other commonly occurring AEs included fever, cough, contusion, dizziness, and febrile neutropenia. Grade 3/4 nausea and grade 3/4 vomiting were each observed in 7% of patients. Grade 3 fatigue was observed in 10% of patients. Diarrhea occurred at grade 3 severity in 10% of patients and grade 4 severity in 2%. Grade 3 febrile neutropenia was observed in eight patients (20%), with four of those having an ANC of ≤ 500/μL at baseline.

Of the 41 patients who received oral azacitidine, 33 terminated from the study as of the date of data analysis, with 17 discontinuing before completing 6 cycles of oral therapy. Reasons for discontinuation included disease progression/treatment failure (n = 10), investigator decision primarily due to absence of observed benefit/response (n = 15), withdrawal of consent (n = 4), AEs (n = 3), and decision to pursue hematopoietic stem-cell transplantation (n = 1). There were three deaths within 28 days of last dose of study drug due to multiple organ failure (n = 1), gastrointestinal hemorrhage (n = 1), and pneumonia plus urinary tract infection (n = 1). No deaths were attributed to study drug. Eight patients remained on the study at the time of data analysis, having each received between 14 and 32 treatment cycles.

Pharmacokinetic Characteristics of Azacitidine

High interpatient variability was noted for all pharmacokinetic parameters. Azacitidine was rapidly absorbed after SC (n = 42) and oral (n = 36) administration, reaching C_{max} within 0.5 hours (range, 0.2 to 1.1 hours) and 1.0 hours (range, 0.3 to 3.6 hours) postdose, respectively. Concentration versus time profiles decreased in a pseudobiphasic manner (Fig 1A). The mean elimination half-life was 1.6 ± 0.7 hours for SC and 0.62 ± 0.25 hours for oral azacitidine. Exposure after single oral administration generally increased with dose (Table 3). For the seven oral dose levels, the mean relative azacitidine oral bioavailability (F) ranged from 6.3% to 20%. The MTD had a mean relative bioavailability of 13% ± 9%. CL/F exceeded hepatic blood flow, indicating extrahepatic metabolism, and Vd/F was greater than total body water, suggesting extensive tissue distribution. The amount of azacitidine recovered in urine relative to dose was small (< 2%) for

Table 3. Day 1 Plasma Pharmacokinetics Parameters After Single Subcutaneous or Oral Azacitidine Administration

Dose	No. of Patients	AUC_{inf} (ng × h/mL) Mean	SD	%CV	CL/F (L/h) Mean	SD	%CV	C_{max} (ng/mL) Mean	SD	%CV	T_{max} (h) Median	Range	Vd/F (L) Mean	SD	%CV	F (%) Mean	SD	Relative Oral Bioavailability
Subcutaneous, 75 mg/m²	42	1,020	440	43*	175	128	73*	650	250	39	0.50	0.2-1.1	410	410	101*	NA		
Oral, mg																		
120	4	62	43	70	4,100	4,860	118	38	24	64	1.48	1.0-2.0	2,930	3,810	130	8.1	5.6	69
180	3	112	64	58	2,330	1,890	81	72	36	50	1.50	1.0-1.5	1,700	1,580	93	6.3	2.3	37
240	3	463	221	48	598	258	43	215	102	47	1.00	1.0-1.5	814	421	52	20.0	9.6	48
300	5	282	88	31	1,180	487	41	144	13	9.2	1.48	1.0-2.0	1,090	626	57	11.5	2.6	23
360	5	311	141	45	1,360	573	42	195	79	40	1.00	0.5-3.6	947	251	27	12.8	2.4	19
480	14	362	253	70	2,140	1,620	76	211	140	66	1.00	0.3-2.5	2,010	1,910	95	12.8	9.4	74†
600	2	502	100	20	1,220	244	20	253	29	12	1.50	1.0-2.0	1,580	1,410	89	14.9	0.8	5

Abbreviations: AUC_{inf}, area under the plasma concentration–time curve from time zero to infinity; CL/F, apparent total clearance; C_{max}, maximum observed plasma concentration; F, relative oral bioavailability; NA, not applicable; T_{max}, time of maximum observed plasma concentration; Vd/F, apparent volume of distribution.
*n = 40.
†n = 13.

SC and oral administration, suggesting that nonrenal elimination is the predominant pathway for clearance. Results after multiple doses were similar to those obtained after a single dose for both administration routes (data not shown). There was no evidence of azacitidine accumulation.

Pharmacodynamics of Azacitidine: Effect on DNA Methylation

DNA methylation was evaluated during cycles 1 and 2 in 10 patients treated with oral azacitidine. The numbers of highly methylated loci were calculated at each time point by averaging across patients the number of loci with methylation ratios ≥ 0.7 (Fig 1B). These numbers decreased after SC and oral administration, with maximal effects at day 15 of each cycle. The reduction in levels of highly methylated loci was not maintained throughout the entire cycle and returned to near-baseline levels by the end of each cycle. SC azacitidine decreased a greater number of loci in comparison to oral azacitidine. The changes in methylation level from baseline across patients for the 5,232 highly methylated loci (average methylation ratio at baseline ≥ 0.7) are represented as box plots (Fig 1C). As with the analysis of total numbers of highly methylated loci, the median DNA methylation of these loci was reduced by 0.115 on day 15 of cycle 1 (SC azacitidine) and 0.055 on day 15 of cycle 2 (oral azacitidine), and returned to baseline levels at the end of each cycle.

Differentially methylated loci at each post-treatment time point compared with baseline were identified in cycles 1 and 2, with the maximum number observed on day 15 of each cycle; 6,981 loci were differentially methylated (6,917 hypomethylated) on day 15 of cycle 1 (SC azacitidine) and 1,609 loci were differentially methylated (1,600 hypomethylated) on day 15 of cycle 2 (oral azacitidine; $P < .01$). In total, 1,482 loci were significantly hypomethylated by both SC and oral azacitidine (Fig 1D), representing 92.6% of all loci significantly hypomethylated by oral azacitidine treatment. These data demonstrate comparable biologic activity with SC and oral azacitidine, albeit to a lesser extent with oral azacitidine.

Clinical Activity of Oral Azacitidine

The median number of oral azacitidine cycles administered to patients with MDSs, CMML, and AML was 6 (range, 1 to 32+), 12.5+ (range, 3 to 28+), and 4.5 (range, 1 to 15), respectively. Treatment duration is summarized in Figure 2. The number of patients from the MDSs, CMML, and AML groups who remained on the study at the

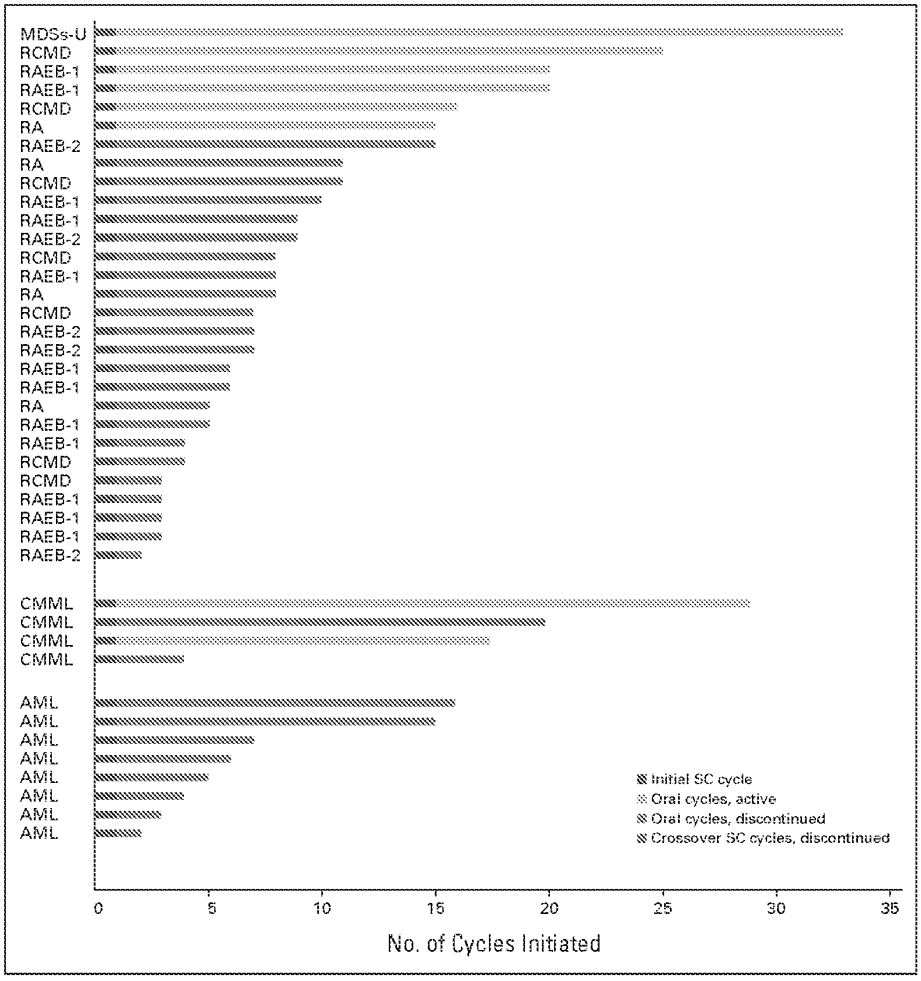

Fig 2. Treatment duration for the 41 patients treated with oral azacitidine (AZA). AML, acute myeloid leukemia; CMML, chronic myelomonocytic leukemia; MDSs-U, myelodysplastic syndromes-unclassified; RA, refractory anemia; RCMD, refractory cytopenias with multilineage dysplasia; RAEB, RA with excess blasts; SC, subcutaneous.

Table 4. Response in Myelodysplastic Syndromes and Chronic Myelomonocytic Leukemia Patients

Response	Previously Treated Patients[a]			First-Line Treatment			Duration of Response, Range (days)
	Responders	Evaluable Patients	%	Responders	Evaluable Patients	%	
Overall response[b]	6	17	35	11	15	73	30-483[c]
CR[d]	0	17	0	6	15	40	30-152
Any HI[e]	6	16	38	5	9	56	56-483[c]
HI-E	3	10	30	2	4	50	56-483[c]
HI-N	0	10	0	2	7	29	82-321[c]
HI-P	5	14	36	2	6	33	58-351[c]
TI	0	5	0	1	3	33	76
Red blood cell	0	3	0	1	3	33	76
Platelet	0	4	0	0			NA
mCR[e,f]	6	9	67	2	6	33	63-422[g]

NOTE. At any cycle of azacitidine, International Working Group 2006 criteria were used with modifications as described in the Patients and Methods section.
Abbreviations: CR, complete remission; E, erythroid; HI, hematologic improvement; mCR, bone marrow complete remission; N, neutrophil; NA, not applicable; P, platelet; TI, transfusion independence

[a]Includes erythropoiesis-stimulating agents, chemotherapy, hypomethylating agents, and investigational and/or other agents.

[b]Overall response rate does not include patients achieving mCR only.

[c]One or more responses, including that at upper limit of range, are ongoing. Data were censored as of last visit entered into the clinical database.

[d]Patients achieving CR were not included in any other categories.

[e]One patient with mCR in the previously treated group also achieved HI (both HI-E and HI-P). Two patients with mCR in the first-line treatment group also achieved HI (one patient with HI-P and one patient with both HI-E and HI-N). These patients have been included in both the mCR and HI categories.

[f]In the eight patients who achieved mCR, the response began in cycle 1 of subcutaneous (SC) dosing (n = 4) or very early in cycle 2 of oral dosing (n = 4). Therefore, the contribution of a single SC azacitidine cycle to the induction of these responses is likely relevant.

[g]Bone marrow aspirates were not required after 6 cycles of oral azacitidine treatment, therefore follow-up data were not available to confirm upper limit of duration. Data were censored as of last visit entered into the clinical database.

time of the analysis was 6, 2, and 0, respectively. Response and duration of response data are summarized in Table 4. In the 17 previously treated patients with MDSs and CMML, the overall response rate was 35%, without including patients who only achieved mCR; if those patients were included the response rate would be 65%. In the 15 patients with MDSs and CMML receiving first-line treatment, the overall response rate was 73% and in this group no patients achieved mCR only. Longest duration of response to date was 483 days overall. In one patient who achieved a CR, the response began before oral dosing and ended in cycle 2, thus was likely attributable to the single cycle of SC azacitidine.

No responses were observed in patients with AML. Two patients with AML (25%) had stable disease for 14 and 15 cycles, respectively, and five patients with AML (63%) received ≥ 4 oral azacitidine cycles.

Discussion

An oral azacitidine formulation may bring advantages for patients (ease of administration), society (health care cost implications), and disease treatment (extended administration), provided that clinical activity and safety are similar to SC/intravenous azacitidine. This phase I trial demonstrated that oral azacitidine is associated with minimal adverse effects at doses lower than 600 mg. The MTD was 480 mg on a 7-day of 28 days treatment schedule. The 600 mg dose was associated with early onset of severe diarrhea in two of three patients. Diarrhea in patients taking oral azacitidine doses lower than 600 mg was self-limiting and manageable by treatment and/or prophylaxis with antidiarrheal agents and/or dose reduction. Azacitidine, along with one or more ingredients used in its formulation, may contribute to the diarrhea observed because it was a common adverse event at most dose levels tested. Gastrointestinal disturbances may have been exacerbated by the requirement to ingest oral azacitidine in a fasting state. Whether oral azacitidine administration with food can reduce gastrointestinal toxicity will be evaluated in ongoing studies. Grade 3 and 4 AEs consisted primarily of febrile neutropenia, gastrointestinal disturbances, and fatigue. Of the eight patients who experienced grade 3 febrile neutropenia, four entered the study with a baseline ANC of $\leq 500/\mu L$.

After oral administration, maximum azacitidine plasma concentrations were achieved rapidly (within 1 hour), suggesting that absorption occurs from the proximal gastrointestinal tract. Azacitidine exposure increased with increasing oral doses, and the mean relative oral bioavailability ranged from 6.3% to 20%. After multiple doses, there was no evidence of azacitidine accumulation, and no apparent decline in absorption was seen between days 1 and 7. Azacitidine clearance was hepatic and extrahepatic, with little evidence of renal clearance.

Kinetics of the change in DNA methylation levels after SC and oral azacitidine were similar, with maximum hypomethylation achieved on day 15, and methylation levels returned to near-baseline values by the end of each cycle. This pattern has been observed in other azacitidine studies.[3,12] At the dosing schedule employed in this study, oral azacitidine affected fewer loci than SC azacitidine; however, 1,482 loci were identified as commonly hypomethylated by both azacitidine formulations.

Significant responses were observed in patients with MDSs and CMML, indicating that oral azacitidine has clinical activity in these settings. Although all patients received an initial cycle of SC azacitidine, which may have contributed to the clinical activity observed, it has been reported that only half of the total hematologic responses to SC azacitidine manifest within 2 cycles.[13] Continued treatment with oral azacitidine following the single cycle of SC azacitidine is therefore likely to be associated with the development and/or maintenance of clinical responses observed in this study.

Results from a study investigating alternative SC azacitidine dosing schedules in lower-risk patients with MDSs suggested that for all

dosing regimens tested, continued azacitidine treatment may be beneficial.[14] The short plasma half-life of azacitidine, S-phase restricted incorporation into DNA, and rapid remethylation of DNA, are contributing factors to the importance of chronic exposure to the drug. It is therefore likely that extended schedules of oral administration will positively affect clinical activity of azacitidine. A follow-up trial has been initiated to investigate the efficacy of such extended schedules.

In conclusion, the MTD for oral azacitidine administered daily for 7 days of a 28-day cycle was determined to be 480 mg, and oral azacitidine is bioavailable and biologically active. Clinical responses were reported in 35% of previously treated patients with MDSs and CMML, and in 73% of patients who received oral azacitidine as first-line therapy. Lower drug exposure and DNA hypomethylation seen with oral azacitidine relative to SC azacitidine provide the rationale for further study of more frequent dosing and extended schedules of oral azacitidine in MDSs, CMML, and AML. While these results show promise for an oral formulation of azacitidine, they are preliminary data and need further research so that these positive early findings can be confirmed in larger numbers of patients.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

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Final approval of manuscript: All authors

REFERENCES

1. Hollenbach PW, Nguyen AN, Brady H, et al: A comparison of azacitidine and decitabine activities in acute myeloid leukemia cell lines. PLoS One 5:e9001, 2010
2. D'Alò F, Voso MT, Leone G: Profile of azacitidine. Therapy 2:717-731, 2005
3. Gore SD, Baylin S, Sugar E, et al: Combined DNA methyltransferase and histone deacetylase inhibition in the treatment of myeloid neoplasms. Cancer Res 66:6361-6369, 2006
4. Stresemann C, Bokelmann I, Mahlknecht U, et al: Azacytidine causes complex DNA methylation responses in myeloid leukemia. Mol Cancer Ther 7:2998-3005, 2008
5. Garcia-Manero G, Stoltz ML, Ward MR, et al: A pilot pharmacokinetic study of oral azacitidine. Leukemia 22:1680-1684, 2008
6. Vardiman JW, Harris NL, Brunning RD: The World Health Organization (WHO) classification of the myeloid neoplasms. Blood 100:2292-2302, 2002
7. Vardiman JW, Thiele J, Arber DA, et al: The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: Rationale and important changes. Blood 114:937-951, 2009
8. Troyanskaya O, Cantor M, Sherlock G, et al: Missing value estimation methods for DNA microarrays. Bioinformatics 17:520-525, 2001
9. Cheson BD, Greenberg PL, Bennett JM, et al: Clinical application and proposal for modification of the International Working Group (IWG) response criteria in myelodysplasia. Blood 103:419-425, 2006
10. Cheson BD, Bennett JM, Kopecky KJ, et al: Revised recommendations of the International Working Group for Diagnosis, Standardization of Response Criteria, Treatment Outcomes, and Reporting Standards for Therapeutic Trials in Acute Myeloid Leukemia. J Clin Oncol 21:4642-4649, 2003
11. Greenberg P, Cox C, LeBeau MM, et al: International scoring system for evaluating prognosis in myelodysplastic syndromes. Blood 89:2079-2088, 1997
12. Soriano AO, Yang H, Faderl S, et al: Safety and clinical activity of the combination of 5-azacytidine, valproic acid, and all-trans retinoic acid in acute myeloid leukemia and myelodysplastic syndrome. Blood 110:2302-2308, 2007
13. Silverman LR, Fenaux P, Mufti GJ, et al: Continued azacitidine therapy beyond time of first response improves quality of response in patients with higher-risk myelodysplastic syndromes. Cancer [Epub ahead of print on January 10, 2011]
14. Lyons RM, Cosgriff TM, Modi SS, et al: Hematologic response to three alternative dosing schedules of azacitidine in patients with myelodysplastic syndromes. J Clin Oncol 27:1850-1856, 2009

HIGHLIGHTS OF PRESCRIBING INFORMATION

These highlights do not include all the information needed to use VIDAZA safely and effectively. See full prescribing information for VIDAZA.

VIDAZA (azacitidine for injection) for SC or IV use
Initial U.S. Approval: 2004

INDICATIONS AND USAGE

VIDAZA is a nucleoside metabolic inhibitor indicated for the treatment of patients with the following FAB myelodysplastic syndrome (MDS) subtypes: Refractory anemia (RA) or refractory anemia with ringed siderobiasts (RARS) (if accompanied by neutropenia or thrombocytopenia or requiring transfusions), refractory anemia with excess blasts (RAEB), refractory anemia with excess blasts in transformation (RAEB-T), and chronic myelomonocytic leukemia (CMMoL). (1)

DOSAGE AND ADMINISTRATION

- The recommended starting dose for the first treatment cycle, for all patients regardless of baseline hematology values, is VIDAZA 75 mg/m² daily for 7 days to be administered by subcutaneous (SC) injection or intravenous (IV) infusion. Premedicate for nausea and vomiting. (2.1)
- Repeat cycles every 4 weeks (2.2). After 2 cycles, may increase dose to 100 mg/m² if no beneficial effect is seen and no toxicity other than nausea and vomiting has occurred (2.2). Patients should be treated for a minimum of 4 to 6 cycles. Complete or partial response may require additional treatment cycles (2.2).
- Continue treatment as long as the patient continues to benefit (2.2).
- Patients should be monitored for hematologic response and renal toxicities, with dosage delay or reduction as appropriate (2.3, 2.4, 2.5).

DOSAGE FORMS AND STRENGTHS

- Lyophilized powder in 100 mg single-use vials (3).

CONTRAINDICATIONS

- Advanced malignant hepatic tumors (4.1).
- Hypersensitivity to azacitidine or mannitol (4.2).

WARNINGS AND PRECAUTIONS

- Anemia, neutropenia and thrombocytopenia. Perform complete blood counts (CBC) prior to each treatment cycle and as needed to monitor response and toxicity. (5.1).
- Hepatotoxicity: Use with caution in patients with severe preexisting liver impairment (5.2).
- Renal abnormalities. Monitor patients with renal impairment for toxicity since azacitidine and its metabolites are primarily excreted by the kidneys (5.3).
- Monitor liver chemistries and serum creatinine prior to initiation of therapy and with each cycle (5.4).
- VIDAZA may cause fetal harm when administered to a pregnant woman. Women of childbearing potential should be apprised of the potential hazard to a fetus. (5.5, 8.1).
- Men should be advised not to father a child while receiving VIDAZA (5.6, 13.1).

ADVERSE REACTIONS

Most common adverse reactions (>30%) by SC route are: nausea, anemia, thrombocytopenia, vomiting, pyrexia, leukopenia, diarrhea, injection site erythema, constipation, neutropenia and ecchymosis. Most common adverse reactions by IV route also included petechiae, rigors, weakness and hypokalemia (6.1).

To report SUSPECTED ADVERSE REACTIONS, contact Celgene Corporation at 1-888-423-5436 or FDA at 1-800-FDA-1088 or *www.fda.gov/medwatch*.

DRUG INTERACTIONS

- No formal clinical assessments of drug-drug interactions between VIDAZA and other agents have been conducted (7).

USE IN SPECIFIC POPULATIONS

- Nursing Mothers: Discontinue drug or nursing taking into consideration importance of drug to mother (8.3).
- Because elderly patients are more likely to have decreased renal function, it may be useful to monitor renal function (8.5).

See 17 for PATIENT COUNSELING INFORMATION.

Revised: December 2012

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FULL PRESCRIBING INFORMATION

1 INDICATIONS AND USAGE

1.1 Myelodysplastic Syndromes (MDS)

VIDAZA® is indicated for treatment of patients with the following French-American-British (FAB) myelodysplastic syndrome subtypes: refractory anemia (RA) or refractory anemia with ringed sideroblasts (if accompanied by neutropenia or thrombocytopenia or requiring transfusions), refractory anemia with excess blasts (RAEB), refractory anemia with excess blasts in transformation (RAEB-T), and chronic myelomonocytic leukemia (CMMoL).

2 DOSAGE AND ADMINISTRATION

2.1 First Treatment Cycle

The recommended starting dose for the first treatment cycle, for all patients regardless of baseline hematology laboratory values, is 75 mg/m² subcutaneously or intravenously, daily for 7 days. Patients should be premedicated for nausea and vomiting.

2.2 Subsequent Treatment Cycles

Cycles should be repeated every 4 weeks. The dose may be increased to 100 mg/m² if no beneficial effect is seen after 2 treatment cycles and if no toxicity other than nausea and vomiting has occurred. It is recommended that patients be treated for a minimum of 4 to 6 cycles. However, complete or partial response may require additional treatment cycles. Treatment may be continued as long as the patient continues to benefit.

Patients should be monitored for hematologic response and renal toxicities *[see Warnings and Precautions (5.3)]*, and dosage delay or reduction as described below may be necessary.

2.3 Dosage Adjustment Based on Hematology Laboratory Values

- For patients with baseline (start of treatment) WBC ≥3.0 x10⁹/L, ANC ≥1.5 x10⁹/L, and platelets ≥75.0 x10⁹/L, adjust the dose as follows, based on nadir counts for any given cycle:

Nadir Counts		% Dose in the Next Course
ANC (x10⁹/L)	Platelets (x10⁹/L)	
<0.5	<25.0	50%
0.5–1.5	25.0-50.0	67%
>1.5	>50.0	100%

- For patients whose baseline counts are WBC <3.0 x10⁹/L, ANC<1.5 x10⁹/L, or platelets <75.0 x10⁹/L, dose adjustments should be based on nadir counts and bone marrow biopsy cellularity at the time of the nadir as noted below, unless there is clear improvement in differentiation (percentage of mature granulocytes is higher and ANC is higher than at onset of that course) at the time of the next cycle, in which case the dose of the current treatment should be continued.

WBC or Platelet Nadir % decrease in counts from baseline	Bone Marrow Biopsy Cellularity at Time of Nadir (%)		
	30-60	15-30	<15
	% Dose in the Next Course		
50 - 75	100	50	33
>75	75	50	33

If a nadir as defined in the table above has occurred, the next course of treatment should be given 28 days after the start of the preceding course, provided that both the WBC and the platelet counts are >25% above the nadir and rising. If a >25% increase above the nadir is not seen by day 28, counts should be reassessed every 7 days. If a 25% increase is not seen by day 42, then the patient should be treated with 50% of the scheduled dose.

2.4 Dosage Adjustment Based on Renal Function and Serum Electrolytes

If unexplained reductions in serum bicarbonate levels to <20 mEq/L occur, the dosage should be reduced by 50% on the next course. Similarly, if unexplained elevations of BUN or serum creatinine occur, the next cycle should be delayed until values return to normal or baseline and the dose should be reduced by 50% on the next treatment course *[see Warnings and Precautions (5.3)]*.

2.5 Use in Geriatric Patients

Azacitidine and its metabolites are known to be substantially excreted by the kidney, and the risk of toxic reactions to this drug may be greater in patients with impaired renal function. Because elderly patients are more likely to have decreased renal function, care should be taken in dose selection, and it may be useful to monitor renal function *[see Warnings and Precautions (5.3) and Use in Specific Populations (8.5)]*.

2.6 Preparation of VIDAZA

VIDAZA is a cytotoxic drug and, as with other potentially toxic compounds, caution should be exercised when handling and preparing VIDAZA suspensions *[see How Supplied/Storage and Handling (16)]*.

If reconstituted VIDAZA comes into contact with the skin, immediately and thoroughly wash with soap and water. If it comes into contact with mucous membranes, flush thoroughly with water.

The VIDAZA vial is single-use and does not contain any preservatives. Unused portions of each vial should be discarded properly *[see How Supplied/Storage and Handling (16)]*. Do not save any unused portions for later administration.

2.7 Instructions for Subcutaneous Administration

VIDAZA should be reconstituted aseptically with 4 mL sterile water for injection. The diluent should be injected slowly into the vial. Vigorously shake or roll the vial until a uniform suspension is achieved. The suspension will be cloudy. The resulting suspension will contain azacitidine 25 mg/mL. Do not filter the suspension after reconstitution. Doing so could remove the active substance.

Preparation for Immediate Subcutaneous Administration: Doses greater than 4 mL should be divided equally into 2 syringes. The product may be held at room temperature for up to 1 hour, but must be administered within 1 hour after reconstitution.

Preparation for Delayed Subcutaneous Administration: The reconstituted product may be kept in the vial or drawn into a syringe. Doses greater than 4 mL should be divided equally into 2 syringes. The product must be refrigerated immediately. When VIDAZA is reconstituted using water for injection that has not been refrigerated, the reconstituted product may be held under refrigerated conditions (2°C - 8°C, 36°F - 46°F) for up to 8 hours. When VIDAZA is reconstituted using refrigerated (2°C - 8°C, 36°F - 46°F) water for injection, the reconstituted product may be stored under refrigerated conditions (2°C - 8°C, 36°F - 46°F) for up to 22 hours. After removal from refrigerated conditions, the suspension may be allowed to equilibrate to room temperature for up to 30 minutes prior to administration.

Subcutaneous Administration

To provide a homogeneous suspension, the contents of the dosing syringe must be re-suspended immediately prior to administration. To re-suspend, vigorously roll the syringe between the palms until a uniform, cloudy suspension is achieved.

VIDAZA suspension is administered subcutaneously. Doses greater than 4 mL should be divided equally into 2 syringes and injected into 2 separate sites. Rotate sites for each injection (thigh, abdomen, or upper arm). New injections should be given at least one inch from an old site and never into areas where the site is tender, bruised, red, or hard.

Suspension Stability: VIDAZA reconstituted with non-refrigerated water for injection for subcutaneous administration may be stored for up to 1 hour at 25°C (77°F) or for up to 8 hours between 2°C and 8°C (36°F and 46°F); when reconstituted with refrigerated (2°C - 8°C, 36°F - 46°F) water for injection, it may be stored for 22 hours between 2°C and 8°C (36°F and 46°F).

2.8 Instructions for Intravenous Administration

Reconstitute the appropriate number of VIDAZA vials to achieve the desired dose. Reconstitute each vial with 10 mL sterile water for injection. Vigorously shake or roll the vial until all solids are dissolved. The resulting solution will contain azacitidine 10 mg/mL. The solution should be clear. Parenteral drug product should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

Withdraw the required amount of VIDAZA solution to deliver the desired dose and inject into a 50 -100 mL infusion bag of either 0.9% Sodium Chloride Injection or Lactated Ringer's Injection.

Intravenous Solution Incompatibility

VIDAZA is incompatible with 5% Dextrose solutions, Hespan, or solutions that contain bicarbonate. These solutions have the potential to increase the rate of degradation of VIDAZA and should therefore be avoided.

Intravenous Administration

VIDAZA solution is administered intravenously. Administer the total dose over a period of 10 - 40 minutes. The administration must be completed within 1 hour of reconstitution of the VIDAZA vial.

Solution Stability: VIDAZA reconstituted for intravenous administration may be stored at 25°C (77°F), but administration must be completed within 1 hour of reconstitution.

3 DOSAGE FORMS AND STRENGTHS

VIDAZA (azacitidine for injection) is supplied as lyophilized powder in 100 mg single-use vials.

4 CONTRAINDICATIONS

4.1 Advanced Malignant Hepatic Tumors

VIDAZA is contraindicated in patients with advanced malignant hepatic tumors *[see Warnings and Precautions (5.2)]*.

4.2 Hypersensitivity to Azacitidine or Mannitol

VIDAZA is contraindicated in patients with a known hypersensitivity to azacitidine or mannitol.

5 WARNINGS AND PRECAUTIONS

5.1 Anemia, Neutropenia and Thrombocytopenia

Treatment with VIDAZA is associated with anemia, neutropenia and thrombocytopenia. Complete blood counts should be performed as needed to monitor response and toxicity, but at a minimum, prior to each dosing cycle. After administration of the recommended dosage for the first cycle, dosage for subsequent cycles should be reduced or delayed based on nadir counts and hematologic response *[see Dosage and Administration (2.3)]*.

5.2 Severe Pre-existing Hepatic Impairment

Because azacitidine is potentially hepatotoxic in patients with severe pre-existing hepatic impairment, caution is needed in patients with liver disease. Patients with extensive tumor burden due to metastatic disease have been reported to experience progressive hepatic coma and death during azacitidine treatment, especially in such patients with baseline albumin <30 g/L. Azacitidine is contraindicated in patients with advanced malignant hepatic tumors *[see Contraindications (4.1)]*.

Safety and effectiveness of VIDAZA in patients with MDS and hepatic impairment have not been studied as these patients were excluded from the clinical trials.

5.3 Renal Abnormalities

Renal abnormalities ranging from elevated serum creatinine to renal failure and death have been reported in patients treated with intravenous azacitidine in combination with other chemotherapeutic agents for nonMDS conditions. In addition, renal tubular acidosis, defined as a fall in serum bicarbonate to <20 mEq/L in association with an alkaline urine and hypokalemia (serum potassium <3 mEq/L) developed in 5 patients with CML treated with azacitidine and etoposide. If unexplained reductions in serum bicarbonate <20 mEq/L or elevations of BUN or serum creatinine occur, the dosage should be reduced or held *[see Dosage and Administration (2.4)]*.

Patients with renal impairment should be closely monitored for toxicity since azacitidine and its metabolites are primarily excreted by the kidneys *[see Dosage and Administration (2.4, 2.5)]*.

Safety and effectiveness of VIDAZA in patients with MDS and renal impairment have not been studied as these patients were excluded from the clinical trials.

5.4 Monitoring Laboratory Tests

Complete blood counts should be performed as needed to monitor response and toxicity, but at a minimum, prior to each cycle. Liver chemistries and serum creatinine should be obtained prior to initiation of therapy.

5.5 Use in Pregnancy

VIDAZA may cause fetal harm when administered to a pregnant woman. Azacitidine caused congenital malformations in animals. Women of childbearing potential should be advised to avoid pregnancy during treatment with VIDAZA. There are no adequate and well-controlled studies in pregnant women using VIDAZA. If this drug is used during pregnancy or if a patient becomes pregnant while taking this drug, the patient should be apprised of the potential hazard to the fetus *[see Use in Specific Populations (8.1)]*.

5.6 Use in Males

Men should be advised to not father a child while receiving treatment with VIDAZA. In animal studies, pre-conception treatment of male mice and rats resulted in increased embryofetal loss in mated females *[see Nonclinical Toxicology (13)]*.

6 ADVERSE REACTIONS

6.1 Overview

Adverse Reactions Described in Other Labeling Sections: anemia, neutropenia, thrombocytopenia, elevated serum creatinine, renal failure, renal tubular acidosis, hypokalemia, hepatic coma *[see Warnings and Precautions (5.1, 5.2, 5.3)]*.

Most Commonly Occurring Adverse Reactions (SC or IV Route): nausea, anemia, thrombocytopenia, vomiting, pyrexia, leukopenia, diarrhea, injection site erythema, constipation, neutropenia, ecchymosis. The most common adverse reactions by IV route also included petechiae, rigors, weakness and hypokalemia.

Adverse Reactions Most Frequently (>2%) Resulting in Clinical Intervention (SC or IV Route):
Discontinuation: leukopenia, thrombocytopenia, neutropenia.
Dose Held: leukopenia, neutropenia, thrombocytopenia, pyrexia, pneumonia, febrile neutropenia.
Dose Reduced: leukopenia, neutropenia, thrombocytopenia.

6.2 Adverse Reactions in Clinical Trials

Because clinical trials are conducted under widely varying conditions, adverse reaction rates observed in the clinical trials of a drug cannot be directly compared to rates in the clinical trials of another drug and may not reflect the rates observed in practice.

The data described below reflect exposure to VIDAZA in 443 MDS patients from 4 clinical studies. Study 1 was a supportive-care controlled trial (SC administration), Studies 2 and 3 were single arm studies (one with SC administration and one with IV administration), and Study 4 was an international randomized trial (SC administration) *[see Clinical Studies (14)]*.

In Studies 1, 2 and 3, a total of 268 patients were exposed to VIDAZA, including 116 exposed for 6 cycles (approximately 6 months) or more and 60 exposed for greater than 12 cycles (approximately one year). VIDAZA was studied primarily in supportive-care controlled and uncontrolled trials (n=150 and n=118, respectively). The population in the subcutaneous studies (n=220) was 23 to 92 years old (mean 66.4 years), 68% male, and 94% white, and had MDS or AML. The population in the IV study (n=48) was 35 to 81 years old (mean 63.1 years), 65% male, and 100% white. Most patients received average daily doses between 50 and 100 mg/m^2.

In Study 4, a total of 175 patients with higher-risk MDS (primarily RAEB and RAEB-T subtypes) were exposed to VIDAZA. Of these patients, 119 were exposed for 6 or more cycles, and 63 for at least 12 cycles. The mean age of this population was 68.1 years (ranging from 42 to 83 years), 74% were male, and 99% were white. Most patients received daily VIDAZA doses of 75 mg/m^2.

Table 1 presents adverse reactions occurring in at least 5% of patients treated with VIDAZA (SC) in Studies 1 and 2. It is important to note that duration of exposure was longer for the VIDAZA-treated group than for the observation group: patients received VIDAZA for a mean of 11.4 months while mean time in the observation arm was 6.1 months.

Table 1: Most Frequently Observed Adverse Reactions (≥ 5.0% in All SC VIDAZA Treated Patients; Studies 1 and 2)

	Number (%) of Patients			
System Organ Class Preferred Term[a]	**All VIDAZA[b] (N=220)**		**Observation[c] (N=92)**	
Blood and lymphatic system disorders				
Anemia	153	(69.5)	59	(64.1)
Anemia aggravated	12	(5.5)	5	(5.4)
Febrile neutropenia	36	(16.4)	4	(4.3)
Leukopenia	106	(48.2)	27	(29.3)
Neutropenia	71	(32.3)	10	(10.9)
Thrombocytopenia	144	(65.5)	42	(45.7)
Gastrointestinal disorders				
Abdominal tenderness	26	(11.8)	1	(1.1)
Constipation	74	(33.6)	6	(6.5)
Diarrhea	80	(36.4)	13	(14.1)
Gingival bleeding	21	(9.5)	4	(4.3)
Loose stools	12	(5.5)	0	
Mouth hemorrhage	11	(5.0)	1	(1.1)
Nausea	155	(70.5)	16	(17.4)
Stomatitis	17	(7.7)	0	
Vomiting	119	(54.1)	5	(5.4)
General disorders and administration site conditions				
Chest pain	36	(16.4)	5	(5.4)
Injection site bruising	31	(14.1)	0	
Injection site erythema	77	(35.0)	0	
Injection site granuloma	11	(5.0)	0	
Injection site pain	50	(22.7)	0	
Injection site pigmentation changes	11	(5.0)	0	
Injection site pruritus	15	(6.8)	0	
Injection site reaction	30	(13.6)	0	
Injection site swelling	11	(5.0)	0	
Lethargy	17	(7.7)	2	(2.2)
Malaise	24	(10.9)	1	(1.1)
Pyrexia	114	(51.8)	28	(30.4)
Infections and infestations				
Nasopharyngitis	32	(14.5)	3	(3.3)
Pneumonia	24	(10.9)	5	(5.4)
Upper respiratory tract infection	28	(12.7)	4	(4.3)
Injury, poisoning, and procedural complications				
Post procedural hemorrhage	13	(5.9)	1	(1.1)
Metabolism and nutrition disorders				
Anorexia	45	(20.5)	6	(6.5)
Musculoskeletal and connective tissue disorders				
Arthralgia	49	(22.3)	3	(3.3)
Chest wall pain	11	(5.0)	0	
Myalgia	35	(15.9)	2	(2.2)
Nervous system disorders				
Dizziness	41	(18.6)	5	(5.4)
Headache	48	(21.8)	10	(10.9)
Psychiatric disorders				
Anxiety	29	(13.2)	3	(3.3)
Insomnia	24	(10.9)	4	(4.3)

continued

Table 1: Most Frequently Observed Adverse Reactions (≥ 5.0% in All SC VIDAZA Treated Patients; Studies 1 and 2)

System Organ Class Preferred Term[a]	Number (%) of Patients	
	All VIDAZA[b] (N=220)	Observation[c] (N=92)
Respiratory, thoracic and mediastinal disorders		
Dyspnea	64 (29.1)	11 (12.0)
Skin and subcutaneous tissue disorders		
Dry skin	11 (5.0)	1 (1.1)
Ecchymosis	67 (30.5)	14 (15.2)
Erythema	37 (16.8)	4 (4.3)
Rash	31 (14.1)	9 (9.8)
Skin nodule	11 (5.0)	1 (1.1)
Urticaria	13 (5.9)	1 (1.1)
Vascular disorders		
Hematoma	19 (8.6)	0
Hypotension	15 (6.8)	2 (2.2)
Petechiae	52 (23.6)	8 (8.7)

[a] Multiple terms of the same preferred terms for a patient are only counted once within each treatment group.
[b] Includes adverse reactions from all patients exposed to VIDAZA, including patients after crossing over from observations.
[c] Includes adverse reactions from observation period only; excludes any adverse events after crossover to VIDAZA.

Table 2 presents adverse reactions occurring in at least 5% of patients treated with VIDAZA in Study 4. Similar to Studies 1 and 2 described above, duration of exposure to treatment with VIDAZA was longer (mean 12.2 months) compared with best supportive care (mean 7.5 months).

Table 2: Most Frequently Observed Adverse Reactions (≥ 5.0% in the VIDAZA Treated Patients and the Percentage with NCI CTC Grade 3/4 Reactions; Study 4)

System Organ Class Preferred Term[a]	Number (%) of Patients			
	Any Grade		Grade 3/4	
	VIDAZA (N=175)	Best Supportive Care Only (N=102)	VIDAZA (N=175)	Best Supportive Care Only (N=102)
Blood and lymphatic system disorders				
Anemia	90 (51.4)	45 (44.1)	24 (13.7)	9 (8.8)
Febrile neutropenia	24 (13.7)	10 (9.8)	22 (12.6)	7 (6.9)
Leukopenia	32 (18.3)	2 (2.0)	26 (14.9)	1 (1.0)
Neutropenia	115 (65.7)	29 (28.4)	107 (61.1)	22 (21.6)
Thrombocytopenia	122 (69.7)	35 (34.3)	102 (58.3)	29 (28.4)
Gastrointestinal disorders				
Abdominal pain	22 (12.6)	7 (6.9)	7 (4.0)	0
Constipation	88 (50.3)	8 (7.8)	2 (1.1)	0
Dyspepsia	10 (5.7)	2 (2.0)	0	0
Nausea	84 (48.0)	12 (11.8)	3 (1.7)	0
Vomiting	47 (26.9)	7 (6.9)	0	0
General disorders and administration site conditions				
Fatigue	42 (24.0)	12 (11.8)	6 (3.4)	2 (2.0)
Injection site bruising	9 (5.1)	0	0	0
Injection site erythema	75 (42.9)	0	0	0
Injection site hematoma	11 (6.3)	0	0	0
Injection site induration	9 (5.1)	0	0	0
Injection site pain	33 (18.9)	0	0	0
Injection site rash	10 (5.7)	0	0	0
Injection site reaction	51 (29.1)	0	1 (0.6)	0
Pyrexia	53 (30.3)	18 (17.6)	8 (4.6)	1 (1.0)
Infections and infestations				
Rhinitis	10 (5.7)	1 (1.0)	0	0
Upper respiratory tract infection	16 (9.1)	4 (3.9)	3 (1.7)	0
Urinary tract infection	15 (8.6)	3 (2.9)	3 (1.7)	0

continued

Table 2: Most Frequently Observed Adverse Reactions (≥ 5.0% in the VIDAZA Treated Patients and the Percentage with NCI CTC Grade 3/4 Reactions; Study 4)

System Organ Class Preferred Term[a]	Number (%) of Patients			
	Any Grade		Grade 3/4	
	VIDAZA (N=175)	Best Supportive Care Only (N=102)	VIDAZA (N=175)	Best Supportive Care Only (N=102)
Investigations				
Weight decreased	14 (8.0)	0	1 (0.6)	0
Metabolism and nutrition disorders				
Hypokalemia	11 (6.3)	3 (2.9)	3 (1.7)	3 (2.9)
Nervous system disorders				
Lethargy	13 (7.4)	2 (2.0)	0	1 (1.0)
Psychiatric disorders				
Anxiety	9 (5.1)	1 (1.0)	0	0
Insomnia	15 (8.6)	3 (2.9)	0	0
Renal and urinary disorders				
Hematuria	11 (6.3)	2 (2.0)	4 (2.3)	1 (1.0)
Respiratory, thoracic and mediastinal disorders				
Dyspnea	26 (14.9)	5 (4.9)	6 (3.4)	2 (2.0)
Dysphea exertional	9 (5.1)	1 (1.0)	0	0
Pharyngolaryngeal pain	11 (6.3)	3 (2.9)	0	0
Skin and subcutaneous tissue disorders				
Erythema	13 (7.4)	3 (2.9)	0	0
Petechiae	20 (11.4)	4 (3.9)	2 (1.1)	0
Pruritus	21 (12.0)	2 (2.0)	0	0
Rash	18 (10.3)	1 (1.0)	0	0
Vascular disorders				
Hypertension	15 (8.6)	4 (3.9)	2 (1.1)	2 (2.0)

[a] Multiple reports of the same preferred term from a patient were only counted once within each treatment.

In Studies 1, 2 and 4 with SC administration of VIDAZA, adverse reactions of neutropenia, thrombocytopenia, anemia, nausea, vomiting, diarrhea, constipation, and injection site erythema/reaction tended to increase in incidence with higher doses of VIDAZA. Adverse reactions that tended to be more pronounced during the first 1 to 2 cycles of SC treatment compared with later cycles included thrombocytopenia, neutropenia, anemia, nausea, vomiting, injection site erythema/pain/bruising/reaction, constipation, petechiae, dizziness, anxiety, hypokalemia, and insomnia. There did not appear to be any adverse reactions that increased in frequency over the course of treatment.

Overall, adverse reactions were qualitatively similar between the IV and SC studies. Adverse reactions that appeared to be specifically associated with the IV route of administration included infusion site reactions (e.g. erythema or pain) and catheter site reactions (e.g. infection, erythema, or hemorrhage).

In clinical studies of either SC or IV VIDAZA, the following serious adverse reactions occurring at a rate of < 5% (and not described in Tables 1 or 2) were reported:

Blood and lymphatic system disorders: agranulocytosis, bone marrow failure, pancytopenia splenomegaly.

Cardiac disorders: atrial fibrillation, cardiac failure, cardiac failure congestive, cardio-respiratory arrest, congestive cardiomyopathy.

Eye disorders: eye hemorrhage

Gastrointestinal disorders: diverticulitis, gastrointestinal hemorrhage, melena, perirectal abscess.

General disorders and administration site conditions: catheter site hemorrhage, general physical health deterioration, systemic inflammatory response syndrome.

Hepatobiliary disorders: cholecystitis.

Immune system disorders: anaphylactic shock, hypersensitivity.

Infections and infestations: abscess limb, bacterial infection, cellulitis, blastomycosis, injection site infection, Klebsiella sepsis, neutropenic sepsis, pharyngitis streptococcal, pneumonia Klebsiella, sepsis, septic shock, Staphylococcal bacteremia, Staphylococcal infection, toxoplasmosis.

Metabolism and nutrition disorders: dehydration.

Musculoskeletal and connective tissue disorders: bone pain aggravated, muscle weakness, neck pain.

Neoplasms benign, malignant and unspecified: leukemia cutis.

Nervous system disorders: cerebral hemorrhage, convulsions, intracranial hemorrhage.

Renal and urinary disorders: loin pain, renal failure.

Respiratory, thoracic and mediastinal disorders: hemoptysis, lung infiltration, pneumonitis, respiratory distress.

Skin and subcutaneous tissue disorders: pyoderma gangrenosum, rash pruritic, skin induration.

Surgical and medical procedures: cholecystectomy.

Vascular disorders: orthostatic hypotension.

6.3 Postmarketing Experience

The following adverse reactions have been identified during postmarketing use of VIDAZA. Because these reactions are reported voluntarily from a population of uncertain size, it is not always possible to reliably estimate their frequency or establish a causal relationship to drug exposure.

- Interstitial lung disease
- Tumor lysis syndrome
- Injection site necrosis
- Sweet's syndrome (acute febrile neutrophilic dermatosis)

7 DRUG INTERACTIONS

No formal clinical assessments of drug-drug interactions between VIDAZA and other agents have been conducted *[see Clinical Pharmacology (12.3)]*.

8 USE IN SPECIFIC POPULATIONS

8.1 Pregnancy

Pregnancy Category D *[see Warning and Precautions (5.5)]*

VIDAZA may cause fetal harm when administered to a pregnant woman. Azacitidine was teratogenic in animals. There are no adequate and well controlled studies with VIDAZA in pregnant women. Women of childbearing potential should be advised to avoid pregnancy during treatment with VIDAZA. If this drug is used during pregnancy or if a patient becomes pregnant while taking this drug, the patient should be apprised of the potential hazard to the fetus.

Female partners of male patients receiving VIDAZA should not become pregnant *[see Nonclinical Toxicology (13.1)]*.

Early embryotoxicity studies in mice revealed a 44% frequency of intrauterine embryonal death (increased resorption) after a single IP (intraperitoneal) injection of 6 mg/m^2 (approximately 8% of the recommended human daily dose on a mg/m^2 basis) azacitidine on gestation day 10. Developmental abnormalities in the brain have been detected in mice given azacitidine on or before gestation day 15 at doses of ~3-12 mg/m^2 (approximately 4%-16% the recommended human daily dose on a mg/m^2 basis).

In rats, azacitidine was clearly embryotoxic when given IP on gestation days 4-8 (postimplantation) at a dose of 6 mg/m^2 (approximately 8% of the recommended human daily dose on a mg/m^2 basis), although treatment in the preimplantation period (on gestation days 1-3) had no adverse effect on the embryos. Azacitidine caused multiple fetal abnormalities in rats after a single IP dose of 3 to 12 mg/m^2 (approximately 8% the recommended human daily dose on a mg/m^2 basis) given on gestation day 9, 10, 11 or 12. In this study azacitidine caused fetal death when administered at 3-12 mg/m^2 on gestation days 9 and 10; average live animals per litter was reduced to 9% of control at the highest dose on gestation day 9. Fetal anomalies included: CNS anomalies (exencephaly/encephalocele), limb anomalies (micromelia, club foot, syndactyly, oligodactyly), and others (micrognathia, gastroschisis, edema, and rib abnormalities).

8.3 Nursing Mothers

It is not known whether azacitidine or its metabolites are excreted in human milk. Because many drugs are excreted in human milk and because of the potential for tumorigenicity shown for azacitidine in animal studies and the potential for serious adverse reactions in nursing infants from VIDAZA, a decision should be made whether to discontinue nursing or to discontinue the drug, taking into consideration the importance of the drug to the mother.

8.4 Pediatric Use

Safety and effectiveness in pediatric patients have not been established.

8.5 Geriatric Use

Of the total number of patients in Studies 1, 2 and 3, 62% were 65 years and older and 21% were 75 years and older. No overall differences in effectiveness were observed between these patients and younger patients. In addition there were no relevant differences in the frequency of adverse reactions observed in patients 65 years and older compared to younger patients.

Of the 179 patients randomized to azacitidine in Study 4, 68% were 65 years and older and 21% were 75 years and older. Survival data for patients 65 years and older were consistent with overall survival results. The majority of adverse reactions occurred at similar frequencies in patients < 65 years of age and patients 65 years of age and older.

Azacitidine and its metabolites are known to be substantially excreted by the kidney, and the risk of adverse reactions to this drug may be greater in patients with impaired renal function. Because elderly patients are more likely to have decreased renal function, it may be useful to monitor renal function *[see Dosage and Administration (2.5) and Warnings and Precautions (5.3)]*.

8.6 Gender

There were no clinically relevant differences in safety and efficacy based on gender.

8.7 Race

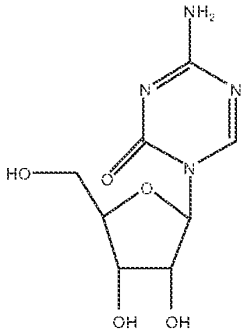
Greater than 90% of all patients in all trials were Caucasian. Therefore, no comparisons between Caucasians and non-Caucasians were possible.

10 OVERDOSAGE

One case of overdose with VIDAZA was reported during clinical trials. A patient experienced diarrhea, nausea, and vomiting after receiving a single IV dose of approximately 290 mg/m^2, almost 4 times the recommended starting dose. The events resolved without sequelae, and the correct dose was resumed the following day. In the event of overdosage, the patient should be monitored with appropriate blood counts and should receive supportive treatment, as necessary. There is no known specific antidote for VIDAZA overdosage.

11 DESCRIPTION

VIDAZA (azacitidine for injection) contains azacitidine, which is a pyrimidine nucleoside analog of cytidine. Azacitidine is 4-amino-1-β-D-ribofuranosyl-s-triazin-2(1H)-one. The structural formula is as follows:



The empirical formula is $C_8H_{12}N_4O_5$. The molecular weight is 244. Azacitidine is a white to off-white solid. Azacitidine was found to be insoluble in acetone, ethanol, and methyl ethyl ketone; slightly soluble in ethanol/water (50/50), propylene glycol, and polyethylene glycol; sparingly soluble in water; water saturated octanol, 5% dextrose in water, N-methyl-2-pyrrolidone, normal saline and 5% Tween 80 in water; and soluble in dimethylsulfoxide (DMSO).

The finished product is supplied in a sterile form for reconstitution as a suspension for subcutaneous injection or reconstitution as a solution with further dilution for intravenous infusion. Vials of VIDAZA contain 100 mg of azacitidine and 100 mg mannitol as a sterile lyophilized powder.

12 CLINICAL PHARMACOLOGY

12.1 Mechanism of Action

VIDAZA is a pyrimidine nucleoside analog of cytidine. VIDAZA is believed to exert its antineoplastic effects by causing hypomethylation of DNA and direct cytotoxicity on abnormal hematopoietic cells in the bone marrow. The concentration of azacitidine required for maximum inhibition of DNA methylation *in vitro* does not cause major suppression of DNA synthesis. Hypomethylation may restore normal function to genes that are critical for differentiation and proliferation. The cytotoxic effects of azacitidine cause the death of rapidly dividing cells, including cancer cells that are no longer responsive to normal growth control mechanisms. Non-proliferating cells are relatively insensitive to azacitidine.

12.3 Pharmacokinetics

The pharmacokinetics of azacitidine were studied in 6 MDS patients following a single 75 mg/m^2 subcutaneous (SC) dose and a single 75 mg/m^2 intravenous (IV) dose. Azacitidine is rapidly absorbed after SC administration; the peak plasma azacitidine concentration of 750 ± 403 ng/ml occurred in 0.5 hour. The bioavailability of SC azacitidine relative to IV azacitidine is approximately 89%, based on area under the curve. Mean volume of distribution following IV dosing is 76 ± 26 L. Mean apparent SC clearance is 167 ± 49 L/hour and mean half-life after SC administration is 41 ± 8 minutes.

Published studies indicate that urinary excretion is the primary route of elimination of azacitidine and its metabolites. Following IV administration of radioactive azacitidine to 5 cancer patients, the cumulative urinary excretion was 85% of the radioactive dose. Fecal excretion accounted for <1% of administered radioactivity over 3 days. Mean excretion of radioactivity in urine following SC administration of ^{14}C-azacitidine was 50%. The mean elimination half-lives of total radioactivity (azacitidine and its metabolites) were similar after IV and SC administrations, about 4 hours.

Special Populations

The effects of renal or hepatic impairment, gender, age, or race on the pharmacokinetics of azacitidine have not been studied *[see Dosage and Administration (2.4), Contraindications (4.1) and Warnings and Precautions (5.2, 5.3)]*.

Drug-Drug Interactions

No formal clinical drug interaction studies with azacitidine have been conducted.

An *in vitro* study of azacitidine incubation in human liver fractions indicated that azacitidine may be metabolized by the liver. Whether azacitidine metabolism may be affected by known microsomal enzyme inhibitors or inducers has not been studied.

An *in vitro* study with cultured human hepatocytes indicated that azacitidine at concentrations up to 100 µM (IV Cmax = 10.6 µM) does not cause any inhibition of CYP2B6 and CYP2C8. The potential of azacitidine to inhibit other cytochrome P450 (CYP) enzymes is not known.

In vitro studies with human cultured hepatocytes indicate that azacitidine at concentrations of 1.0 µM to 100 µM does not induce CYP 1A2, 2C19, or 3A4/5.

13 NONCLINICAL TOXICOLOGY

13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility

The potential carcinogenicity of azacitidine was evaluated in mice and rats. Azacitidine induced tumors of the hematopoietic system in female mice at 2.2 mg/kg (6.6 mg/m², approximately 8% the recommended human daily dose on a mg/m² basis) administered IP three times per week for 52 weeks. An increased incidence of tumors in the lymphoreticular system, lung, mammary gland, and skin was seen in mice treated with azacitidine IP at 2.0 mg/kg (6.0 mg/m², approximately 8% the recommended human daily dose on a mg/m² basis) once a week for 50 weeks. A tumorigenicity study in rats dosed twice weekly at 15 or 60 mg/m² (approximately 20-80% the recommended human daily dose on a mg/m² basis) revealed an increased incidence of testicular tumors compared with controls.

The mutagenic and clastogenic potential of azacitidine was tested in *in vitro* bacterial systems Salmonella typhimurium strains TA100 and several strains of trpE8, Escherichia coli strains WP14 Pro, WP3103P, WP3104P, and CC103; in *in vitro* forward gene mutation assay in mouse lymphoma cells and human lymphoblast cells; and in an *in vitro* micronucleus assay in mouse L5178Y lymphoma cells and Syrian hamster embryo cells. Azacitidine was mutagenic in bacterial and mammalian cell systems. The clastogenic effect of azacitidine was shown by the induction of micronuclei in L5178Y mouse cells and Syrian hamster embryo cells.

Administration of azacitidine to male mice at 9.9 mg/m² (approximately 9% the recommended human daily dose on a mg/m² basis) daily for 3 days prior to mating with untreated female mice resulted in decreased fertility and loss of offspring during subsequent embryonic and postnatal development. Treatment of male rats 3 times per week for 11 or 16 weeks at doses of 15-30 mg/m² (approximately 20-40%, the recommended human daily dose on a mg/m² basis) resulted in decreased weight of the testes and epididymides, and decreased sperm counts accompanied by decreased pregnancy rates and increased loss of embryos in mated females. In a related study, male rats treated for 16 weeks at 24 mg/m² resulted in an increase in abnormal embryos in mated females when examined on day 2 of gestation.

14 CLINICAL STUDIES

Myelodysplastic Syndromes (MDS)

Study 1 was a randomized, open-label, controlled trial carried out in 53 U.S. sites compared the safety and efficacy of subcutaneous VIDAZA plus supportive care with supportive care alone ("observation") in patients with any of the five FAB subtypes of myelodysplastic syndromes (MDS): refractory anemia (RA), RA with ringed sideroblasts (RARS), RA with excess blasts (RAEB), RAEB in transformation (RAEB-T), and chronic myelomonocytic leukemia (CMMoL). RA and RARS patients were included if they met one or more of the following criteria: required packed RBC transfusions; had platelet counts ≤50.0 x 10⁹/L; required platelet transfusions; or were neutropenic (ANC <1.0 x 10⁹/L) with infections requiring treatment with antibiotics. Patients with acute myelogenous leukemia (AML) were not intended to be included. Supportive care allowed in this study included blood transfusion products, antibiotics, antiemetics, analgesics and antipyretics. The use of hematopoeitic growth factors was prohibited. Baseline patient and disease characteristics are summarized in Table 3; the 2 groups were similar.

VIDAZA was administered at a subcutaneous dose of 75 mg/m² daily for 7 days every 4 weeks. The dose was increased to 100 mg/m² if no beneficial effect was seen after 2 treatment cycles. The dose was decreased and/or delayed based on hematologic response or evidence of renal toxicity. Patients in the observation arm were allowed by protocol to cross over to VIDAZA if they had increases in bone marrow blasts, decreases in hemoglobin, increases in red cell transfusion requirements, or decreases in platelets, or if they required a platelet transfusion or developed a clinical infection requiring treatment with antibiotics. For purposes of assessing efficacy, the primary endpoint was response rate (as defined in Table 4).

Of the 191 patients included in the study, independent review (adjudicated diagnosis) found that 19 had the diagnosis of AML at baseline. These patients were excluded from the primary analysis of response rate, although they were included in an intent-to-treat (ITT) analysis of all patients randomized. Approximately 55% of the patients randomized to observation crossed over to receive VIDAZA treatment.

Table 3. Baseline Demographics and Disease Characteristics

	VIDAZA (N=99)	Observation (N=92)
Gender (n%)		
Male	72 (72.7)	60 (65.2)
Female	27 (27.3)	32 (34.8)
Race (n%)		
White	93 (93.9)	85 (92.4)
Black	1 (1.0)	1 (1.1)
Hispanic	3 (3.0)	5 (5.4)
Asian/Oriental	2 (2.0)	1 (1.1)
Age (years)		
N	99	91
Mean ± SD	67.3 ± 10.39	68.0 ± 10.23
Range	31 - 92	35 - 88
Adjudicated MDS diagnosis at study entry (n%)		
RA	21 (21.2)	18 (19.6)
RARS	6 (6.1)	5 (5.4)
RAEB	38 (38.4)	39 (42.4)
RAEB-T	16 (16.2)	14 (15.2)
CMMoL	8 (8.1)	7 (7.6)
AML	10 (10.1)	9 (9.8)
Transfusion product used in 3 months before study entry (n%)		
Any transfusion product	70 (70.7)	59 (64.1)
Blood cells, packed human	66 (66.7)	55 (59.8)
Platelets, human blood	15 (15.2)	12 (13.0)
Hetastarch	0(0.0)	1(1.1)
Plasma protein fraction	1(1.0)	0(0.0)
Other	2(2.0)	2(2.2)

Table 4. Response Criteria

		RA	RARS	RAEB	RAEB-T	CMMoL
Complete Response (CR), duration ≥4 weeks	**Marrow**	<5% blasts				
	Peripheral Blood	Normal CBC if abnormal at baseline Absence of blasts in the peripheral circulation				
Partial Response (PR), duration ≥4 weeks	**Marrow**	No marrow requirements		≥50% decrease in blasts Improvement of marrow dyspoiesis		
	Peripheral Blood	≥50% restoration in the deficit from normal levels of baseline white cells, hemoglobin and platelets if abnormal at baseline No blasts in the peripheral circulation For CMMoL, if WBC is elevated at baseline, a ≥75% reduction in the excess count over the upper limit of normal				

The overall response rate (CR + PR) of 15.7% in VIDAZA-treated patients without AML (16.2% for all VIDAZA randomized patients including AML) was statistically significantly higher than the response rate of 0% in the observation group (p<0.0001) (Table 5). The majority of patients who achieved either CR or PR had either 2 or 3 cell line abnormalities at baseline (79%; 11/14) and had elevated bone marrow blasts or were transfusion dependent at baseline. Patients responding to VIDAZA had a decrease in bone marrow blasts percentage, or an increase in platelets, hemoglobin or WBC. Greater than 90% of the responders initially demonstrated these changes by the 5th treatment cycle. All patients who had been transfusion dependent became transfusion independent during PR or CR. The mean and median duration of clinical response of PR or better was estimated as 512 and 330 days, respectively; 75% of the responding patients were still in PR or better at completion of treatment. Response occurred in all MDS subtypes as well as in patients with adjudicated baseline diagnosis of AML.

Table 5. Response Rates

	VIDAZA (N=89)	Observation Before Crossover (N=83)	
Response	**n (%)**	**n (%)**	**P value**
Overall (CR+PR)	14 (15.7)	0 (0.0)	(<0.0001)
Complete (CR)	5 (5.6)	0 (0.0)	(0.06)
Partial (PR)	9 (10.1)	0 (0.0)	-

Patients in the observation group who crossed over to receive VIDAZA treatment (47 patients) had a response rate of 12.8%.

Study 2, a multi-center, open-label, single-arm study of 72 patients with RAEB, RAEB-T, CMMoL, or AML was also carried out. Treatment with subcutaneous VIDAZA resulted in a response rate (CR + PR) of 13.9%, using criteria similar to those described above. The mean and median duration of clinical response of PR or better was estimated as 810 and 430 days, respectively; 80% of the responding patients were still in PR or better at the time of completion of study involvement. In Study 3, another open-label, single-arm study of 48 patients with RAEB, RAEB-T, or AML, treatment with intravenous VIDAZA resulted in a response rate of 18.8%, again using criteria similar to those described above. The mean and median duration of clinical response of PR or better was estimated as 389 and 281 days, respectively; 67% of the responding patients were still in PR or better at the time of completion of treatment. Response occurred in all MDS subtypes as well as in patients with adjudicated baseline diagnosis of AML in both of these studies. VIDAZA dosage regimens in these 2 studies were similar to the regimen used in the controlled study.

Benefit was seen in patients who did not meet the criteria for PR or better, but were considered "improved." About 24% of VIDAZA-treated patients were considered improved, and about 2/3 of those lost transfusion dependence. In the observation group, only 5/83 patients met criteria for improvement; none lost transfusion dependence. In all 3 studies, about 19% of patients met criteria for improvement with a median duration of 195 days.

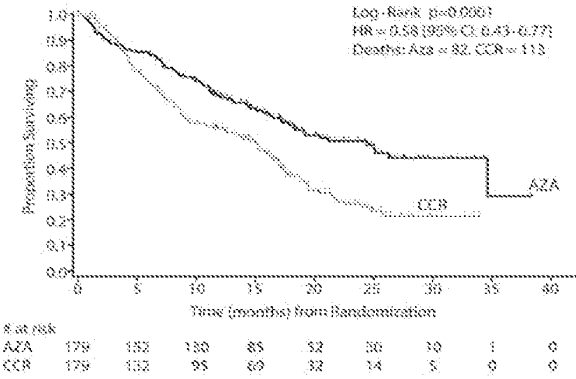
Study 4 was an international, multicenter, open-label, randomized trial in MDS patients with RAEB, RAEB-T or modified CMMoL according to FAB classification and Intermediate-2 and High risk according to IPSS classification. Of the 358 patients enrolled in the study, 179 were randomized to receive azacitidine plus best supportive care (BSC) and 179 were randomized to receive conventional care regimens (CCR) plus BSC (105 to BSC alone, 49 to low dose cytarabine and 25 to chemotherapy with cytarabine and anthracycline). The primary efficacy endpoint was overall survival.

The azacitidine and CCR groups were comparable for baseline parameters. The median age of patients was 69 years (range was 38-88 years), 98% were Caucasian, and 70% were male. At baseline, 95% of the patients were higher risk by FAB classification: RAEB (58%), RAEB-T (34%), and CMMoL (3%). By IPSS classification, 87% were higher risk: Int-2 (41%), High (47%). At baseline, 32% of patients met WHO criteria for AML.

Azacitidine was administered subcutaneously at a dose of 75 mg/m^2 daily for 7 consecutive days every 28 days (which constituted one cycle of therapy). Patients continued treatment until disease progression, relapse after response, or unacceptable toxicity. Azacitidine patients were treated for a median of 9 cycles (range 1 to 39), BSC only patients for a median of 7 cycles (range 1 to 26), low dose cytarabine patients for a median of 4.5 cycles (range 1 to 15), and chemotherapy with cytarabine and anthracycline patients for a median of 1 cycle (range 1 to 3, i.e. induction plus 1 or 2 consolidation cycles).

In the Intent-to-Treat analysis, patients treated with azacitidine demonstrated a statistically significant difference in overall survival as compared to patients treated with CCR (median survival of 24.5 months vs. 15.0 months; stratified log-rank p=0.0001). The hazard ratio describing this treatment effect was 0.58 (95% CI: 0.43, 0.77).

Kaplan-Meier Curve of Time to Death from Any Cause: (Intent-to-Treat Population)



Key: AZA = azacitidine; CCR = conventional care regimens; CI = confidence interval; HR = Hazard Ratio

Azacitidine treatment led to a reduced need for red blood cell transfusions (see Table 6). In patients treated with azacitidine who were RBC transfusion dependent at baseline and became transfusion independent, the median duration of RBC transfusion independence was 13.0 months.

Table 6. Effect of Azacitidine on RBC Transfusions in MDS Patients

Efficacy Parameter	Azacitidine plus BSC (n= 179)	Conventional Care Regimens (n= 179)
Number and percent of patients who were transfusion dependent at baseline who became transfusion independent on treatment[1]	50/111 (45.0%) (95% CI: 35.6%, 54.8%)	13/114 (11.4%) (95% CI: 6.2%, 18.7%)
Number and percent of patients who were transfusion-independent at baseline who became transfusion-dependent on treatment	10/68 (14.7%) (95% CI: 7.3%, 25.4%)	28/65 (43.1%) (95% CI: 30.9%, 56.0%)

[1] A patient was considered RBC transfusion independent during the treatment period if the patient had no RBC transfusions during any 56 consecutive days or more during the treatment period. Otherwise, the patient was considered transfusion dependent.

15 REFERENCES

1. Preventing Occupational Exposures to Antineoplastic and Other Hazardous Drugs in Health Care Settings. NIOSH Alert 2004-165.
2. OSHA Technical Manual, TED 1-0.15A, Section VI: Chapter 2. Controlling Occupational Exposure to Hazardous Drugs. OSHA, 1999. http://www.osha.gov/dts/osta/otm/otm_vi/otm_vi_2.html
3. American Society of Health-System Pharmacists. ASHP guidelines on handling hazardous drugs. Am J Health-Syst Pharm. (2006) 63:1172-1193.
4. Polovich, M., White, J. M., & Kelleher, L.O. (eds.) 2005. Chemotherapy and biotherapy guidelines and recommendations for practice (2nd. ed.) Pittsburgh, PA: Oncology Nursing Society.

16 HOW SUPPLIED/STORAGE AND HANDLING

How Supplied

VIDAZA (azacitidine for injection) is supplied as a lyophilized powder in 100 mg single-use vials packaged in cartons of 1 vial (NDC 59572-102-01).

Storage

Store unreconstituted vials at 25° C (77° F); excursions permitted to 15°-30° C (59°-86° F) (See USP Controlled Room Temperature).

Handling and Disposal

Procedures for proper handling and disposal of anticancer drugs should be applied. Several guidelines on this subject have been published.[1-4] There is no general agreement that all of the procedures recommended in the guidelines are necessary or appropriate.

17 PATIENT COUNSELING INFORMATION

Instruct patients to inform their physician about any underlying liver or renal disease.

Advise women of childbearing potential to avoid becoming pregnant while receiving treatment with VIDAZA. For nursing mothers, a decision should be made whether to discontinue nursing or to discontinue the drug, taking into consideration the importance of the drug to the mother.

Advise men not to father a child while receiving treatment with VIDAZA.

Manufactured for: Celgene Corporation
Summit, NJ 07901

Manufactured by: Ben Venue Laboratories, Inc.
Bedford, OH 44146

Or

Baxter Oncology GmbH
33790 Halle/Westfalen Germany

Or

BSP Pharmaceuticals S.r.l.
04013 Latina Scalo (LT)
Italy

VIDPI.007 12/12

Electronic Patent Application Fee Transmittal

Application Number:	12466213
Filing Date:	14-May-2009
Title of Invention:	ORAL FORMULATIONS OF CYTIDINE ANALOGS AND METHODS OF USE THEREOF
First Named Inventor/Applicant Name:	Jeffrey B. Etter
Filer:	Robert Chang/Carrie Hines
Attorney Docket Number:	9516-847-999/501872-847

Filed as Large Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Extension - 3 months with $0 paid	1253	1	1400	1400

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
Submission- Information Disclosure Stmt	1806	1	180	180
		Total in USD ($)		**1580**

Electronic Acknowledgement Receipt

EFS ID:	18328376
Application Number:	12466213
International Application Number:	
Confirmation Number:	5370
Title of Invention:	ORAL FORMULATIONS OF CYTIDINE ANALOGS AND METHODS OF USE THEREOF
First Named Inventor/Applicant Name:	Jeffrey B. Etter
Customer Number:	84802
Filer:	Robert Chang/Eric Baclig
Filer Authorized By:	Robert Chang
Attorney Docket Number:	9516-847-999/501872-847
Receipt Date:	27-FEB-2014
Filing Date:	14-MAY-2009
Time Stamp:	19:30:13
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$1580
RAM confirmation Number	6498
Deposit Account	503013
Authorized User	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)

1		9516-847-999_Amendment.pdf	3075142 a0be842c6e9fcc2fee0e95c6097b123d396353f1	yes	68

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Amendment/Req. Reconsideration-After Non-Final Reject	1	1
	Claims	2	7
	Applicant Arguments/Remarks Made in an Amendment	8	23
	Affidavit-traversing rejectns or objectns rule 132	24	68

Warnings:

Information:

2	Transmittal Letter	9516-847-999_IDS_Transmittal.pdf	56016 f90af09bb217d8ff629122298c6cd4864912c70a	no	2

Warnings:

Information:

3	Information Disclosure Statement (IDS) Form (SB08)	9516-847-999_IDS.pdf	528158 975ad24380d68275f7b1297f8a724cb1c6c70b75	no	4

Warnings:

Information:

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4	Non Patent Literature	Garcia-Manero_2012_Abstract_424.pdf	217733 8919777110e5e6b981f9cc417f756c98d33145e0	no	4

Warnings:

Information:

5	Non Patent Literature	Garcia-Manero_2011_JClinOncol_2521-2527.pdf	150317 2f087fdfe72944c0469baf0b133411c603f7c2de	no	7

Warnings:

Information:

6	Non Patent Literature	Vidaza_2012_PrescribingInformation_Dec2012.pdf	88773 952898c0b111dc2621dcca1f1586a45bf17f5404	no	7

Warnings:

Information:

7	Fee Worksheet (SB06)	fee-info.pdf	32445 e7e05771899236e5558f6320248c654737302554	no	2
Warnings:					
Information:					
			Total Files Size (in bytes):	4148584	

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

ELECTRONIC FILING

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	Jeffrey B. Etter	Confirmation No.:	5370
Application No.:	12/466,213	Art Unit:	1673
Filed:	May 14, 2009	Examiner:	Lawrence E. Crane
For:	ORAL FORMULATIONS OF CYTIDINE ANALOGS AND METHODS OF USE THEREOF	Attorney Docket (CAM No.:	9516-847-999 501872-999847)

AMENDMENT AND RESPONSE

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

In response to the Non-final Office Action mailed August 29, 2013, Applicants respectfully submit the following amendments and remarks for the Examiner's consideration and entry into the record. Applicants also submit herewith (i) a Declaration under 37 C.F.R. § 1.132 with Exhibits; and (ii) and a supplemental Information Disclosure Statement. Applicants request an Extension of Time for three months from November 29, 2013 to and including February 28, 2014 for the filing of this paper.

Amendments to the Claims are reflected in the listing of the claims that begins on page 2 of this paper.

Remarks begin on page 8 of this paper.

AMENDMENTS TO THE CLAIMS

The following listing of claims will replace all prior versions, and listings, of claims in the application.

Listing of the Claims

1. (Currently Amended) A pharmaceutical composition for oral administration comprising a therapeutically effective amount of 5-azacytidine and at least one pharmaceutically acceptable excipient, wherein the composition is ~~an immediate release~~ a non-enteric coated tablet ~~or an immediate release capsule~~.

2-5. (Canceled).

6. (Previously presented) The composition of claim 1, wherein the at least one pharmaceutically acceptable excipient is selected from mannitol, microcrystalline cellulose, crospovidone, and magnesium stearate.

7. (Original) The composition of claim 1, which further comprises a permeation enhancer.

8. (Previously presented) The composition of claim 7, wherein the permeation enhancer is D-alpha-tocopheryl polyethylene glycol 1000 succinate.

9. (Previously presented) The composition of claim 8, wherein the D-alpha-tocopheryl polyethylene glycol 1000 succinate is present in the composition at about 2% by weight relative to the total weight of the composition.

10. (Original) The composition of claim 1, which is essentially free of a cytidine deaminase inhibitor.

11. (Original) The composition of claim 1, which is essentially free of tetrahydrouridine.

12. (Canceled).

13. (Previously presented) The composition of claim 1, wherein the amount of 5-azacytidine is at least about 40 mg.

14. (Previously presented) The composition of claim 1, wherein the amount of 5-azacytidine is at least about 400 mg.

15. (Previously presented) The composition of claim 1, wherein the amount of 5-azacytidine is at least about 1000 mg.

16. (Previously presented) The composition of claim 1, which has been shown to achieve an area-under-the-curve value of at least about 200 ng-hr/mL following oral administration to a test subject.

17. (Previously presented) The composition of claim 1, which has been shown to achieve an area-under-the-curve value of at least about 400 ng-hr/mL following oral administration to a test subject.

18. (Previously presented) The composition of claim 1, which has been shown to achieve a maximum plasma concentration of at least about 100 ng/mL following oral administration to a test subject.

19. (Previously presented) The composition of claim 1, which has been shown to achieve a maximum plasma concentration of at least about 200 ng/mL following oral administration to a test subject.

20. (Previously presented) The composition of claim 1, which has been shown to achieve a time to maximum plasma concentration of less than about 180 minutes following oral administration to a test subject.

21. (Previously presented) The composition of claim 1, which has been shown to achieve a time to maximum plasma concentration of less than about 90 minutes following oral administration to a test subject.

22. (Previously presented) The composition of claim 1, which has been shown to achieve a time to maximum plasma concentration of less than about 60 minutes following oral administration to a test subject.

23. (Currently Amended) A method for treating one or more symptoms of a disease associated with abnormal cell proliferation, comprising orally administering to a subject in need thereof a pharmaceutical composition comprising a therapeutically effective amount of 5-azacytidine and at least one pharmaceutically acceptable excipient, wherein the composition ~~releases the 5-azacytidine substantially in the stomach following oral administration~~ is a non-enteric coated tablet ~~to the subject~~, and wherein the disease associated with abnormal cell proliferation is myelodysplastic syndrome or acute myelogenous leukemia.

24-32. (Canceled).

33. (Original) The method of claim 23, wherein the composition further comprises a permeation enhancer.

34. (Previously presented) The method of claim 33, wherein the permeation enhancer is D-alpha-tocopheryl polyethylene glycol 1000 succinate.

35. (Previously presented) The method of claim 34, wherein the D-alpha-tocopheryl polyethylene glycol 1000 succinate is present in the formulation at about 2% by weight relative to the total weight of the formulation.

36. (Previously presented) The method of claim 23, wherein the method further comprises not co-administering a cytidine deaminase inhibitor with the 5-azacytidine.

37. (Original) The method of claim 23, wherein the composition is a single unit dosage form.

38-40. (Canceled).

41. (Previously presented) The method of claim 23, wherein the at least one pharmaceutically acceptable excipient is selected from mannitol, microcrystalline cellulose, crospovidone, and magnesium stearate.

42. (Previously presented) The method of claim 23, wherein the amount of 5-azacytidine is at least about 40 mg.

43. (Previously presented) The method of claim 23, wherein the amount of 5-azacytidine is at least about 400 mg.

44. (Previously presented) The method of claim 23, wherein the amount of 5-azacytidine is at least about 1000 mg.

45. (Previously presented) The method of claim 23, which has been shown to achieve an area-under-the-curve value of at least about 200 ng-hr/mL following oral administration to a test subject.

46. (Previously presented) The method of claim 23, which has been shown to achieve an area-under-the-curve value of at least about 400 ng-hr/mL following oral administration to a test subject.

47. (Previously presented) The method of claim 23, which has been shown to achieve a maximum plasma concentration of at least about 100 ng/mL following oral administration to a test subject.

48. (Previously presented) The method of claim 23, which has been shown to achieve a maximum plasma concentration of at least about 200 ng/mL following oral administration to a test subject.

49. (Previously presented) The method of claim 23, which has been shown to achieve a time to maximum plasma concentration of less than about 180 minutes following oral administration to a test subject.

50. (Previously presented) The method of claim 23, which has been shown to achieve a time to maximum plasma concentration of less than about 90 minutes following oral administration to a test subject.

51-72. (Canceled).

73. (New) The composition of claim 1, wherein the tablet comprises a sugar coating, a film coating, or a compression coating.

74. (New) The composition of claim 73, wherein the film coating is a cellulose ether polymer.

75. (New) The composition of claim 74, wherein the cellulose ether polymer is hydroxypropyl methyl-cellulose, hydroxypropyl cellulose, or methyl-cellulose.

76. (New) The composition of claim 1, wherein the amount of 5-azacytidine is 40 to 480 mg.

77. (New) The composition of claim 1, wherein the amount of 5-azacytidine is 80 to 480 mg.

78. (New) The composition of claim 1, wherein the amount of 5-azacytidine is 120 to 480 mg.

79. (New) The composition of claim 1, wherein the amount of 5-azacytidine is at least about 300 mg.

80. (New) The composition of claim 1, wherein the amount of 5-azacytidine is at least about 360 mg.

81. (New) The composition of claim 1, wherein the amount of 5-azacytidine is at least about 400 mg.

82. (New) The composition of claim 1, wherein the amount of 5-azacytidine is at least about 480 mg.

REMARKS

Prior to entry of this paper, claims 1, 3-23, 31-55, 57, 59-61 and 72 were pending in this application. Claims 3-5, 12, 31, 32, 38-40, 51-55, 57, 59-61, and 72 are canceled herein without prejudice to Applicants' right to pursue the subject matter of the canceled claims in this application or a related application. Claims 1 and 23 have been amended without any intention of disclaiming any equivalents thereof. New claims 73-82 have been added.

Specifically, claims 1 and 23 are amended to recite that the composition is a non-enteric coated tablet. Support for this amendment can be found, for example, in original claim 3. New claims 73-75 depend on claim 1 and further recite sugar, film, and compression coatings. Support for these new claims can be found, for example, in paragraphs [0133], [0134], and [0135] of the specification. New claims 76-82 depend on claim 1 and recite specific amounts of 5-azacytidine. Support for these new claims can be found, for example, in paragraph [0123] of the specification. No new matter is introduced.

Applicants respectfully submit that the pending claims are allowable for at least the following reasons.

I. Applicants' Statement of Substance of Interview

An interview was held on January 29, 2014, between Examiner Lawrence E. Crane and C.L. Beach, Donna Robertson-Chow, Yeah-Sil Moon, and Robert Chang, Representatives for Applicants. Applicants thank the Examiner for the courtesy that he extended during the interview.

During the interview, the patentability of the pending claims in view of documents cited by the Examiner was discussed. The Examiner stated that the presentation by Applicants' representatives was useful and requested that Applicants submit a declaration under 37 C.F.R. § 1.132 including the points presented at the interview regarding the rejections under 35 U.S.C. § 112 and § 103. Accordingly, Applicants are concurrently filing a Declaration by Charles L. Beach, Ph.D. under 37 C.F.R. §1.132 ("the Beach Declaration"), as requested by the Examiner.

II. The Rejection Under 35 U.S.C. § 112, First Paragraph, Should Be Withdrawn

Claims 1, 3-23, 31-55, 57, 59-61 and 72 are rejected under 35 U.S.C. § 112, first paragraph, as allegedly not being enabled. (Office Action, page 2.) The Office acknowledges that the specification is enabled for treatment by the administration of 5-azacytidine as the sole active ingredient, but alleges that the specification does not provide enablement for a second "anti-cancer agent" or pharmaceutical compositions comprising an "immediate release" capability. (*Id.*) Applicants respectfully disagree with this rejection.

The test of enablement is whether one reasonably skilled in the art could make or use the invention from the disclosures in the patent coupled with information known in the art without undue experimentation. *U.S. v. Telectronics, Inc.*, 857 F.2d 778, 785 (Fed. Cir. 1988). "A specification disclosure...must be taken as being in compliance with the enablement requirement...unless there is a reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support." (*Id.*)

At the outset, Applicants have deleted the term "immediate release" from the instant claims without acquiescing to the rejection and solely to expedite the allowance of the present application. Thus, to the extent the enablement rejection under 35 U.S.C. § 112, first paragraph, is based on the term "immediate release," the rejection is moot and should be withdrawn.

With regard to the use of a second "anti-cancer agent," Applicants respectfully submit that the specification, coupled with information known in the art, would have enabled a skilled artisan to treat the recited disorders using the claimed compositions and a second anti-cancer agent. First, as acknowledged by the Office, the specification meets the enablement requirement for "the treatment by administration of 5-azacytidine as the sole active ingredient...." (Office Action, page 2.) Second, a skilled artisan is also able to readily formulate the claimed tablet using customary tablet excipients and tableting techniques known in the art. Moreover, a detailed description of excipients and how to make the recited tablets is provided in Section C

beginning on page 17 of the specification[1]. Third, the specification provides exemplary anti-cancer agents, *e.g.*, in paragraphs [00167] and [00168]. With the claimed compositions and active agents in hand, one need only determine the optimal administration regimens for the combination treatment, which a skilled artisan is prepared to do. *See, e.g., Ex parte Skuballa*, 12 U.S.P.Q.2d 1570 (Bd. Pat. App. & Interf. 1989) ("We are satisfied that the skilled worker in this art could readily optimize effective dosages and administration regimens...."); *see also In re Wands*, 858 F.2d 731, 740 (Fed. Cir. 1988) (claims directed to immunoassay methods held to be enabled - even though screening step required - because "[p]ractitioners in this art are prepared to screen negative hybridomas).

For at least the reasons set forth above, Applicants respectfully submit that the rejection under 35 U.S.C. § 112, first paragraph, should be withdrawn.

III. The Claim Rejections Under 35 U.S.C. § 112, Second Paragraph, Should Be Withdrawn

Claims 1, 3-23, 31-55, 57, 59-61 and 72, are rejected under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite. (Office Action, page 4.)

With respect to claim 1, the Office alleges that the phrase "at least one pharmaceutically acceptable excipient" in claim 1 appears to be inconsistent with the term "immediate release tablet" or "immediate release capsule" because the term "immediate release" implies the presence of "more than one excipients." (Office Action at 4.) The Office also alleges that this claim is incomplete because the entire contents of the tablet have been defined functionally by the term "immediate release." (*Id.*)

For at least the reasons stated in Applicants' response filed on August 3, 2013, the entirety of which is incorporated herein by reference, Applicants respectfully submit that this rejection is not proper. Nevertheless, without acquiescing to the rejection and solely to expedite the prosecution of the present application, the term "immediate release" has been deleted from claim 1. Thus, to the extent the rejection under 35 U.S.C. § 112, second paragraph, is based on

[1] Page and paragraph numbered cited herein refer to those of the corresponding published application, U.S. Patent Publication No. 2009/0286752

the term "immediate release, the rejection is moot and should be withdrawn.

With respect to claims 12, 31, 57, and 72, the Office alleges that the term "an additional therapeutic agent"[2] renders the claim indefinite. (*Id.*) Without acquiescing to the rejection and solely to expedite the prosecution of this case, claims 12, 31, 57, and 72 have been canceled. Thus, to the extent the rejection under 35 U.S.C. § 112, second paragraph, is based on the term "additional therapeutic agent," the rejection of claims 12, 31, 57, and 72 under 35 U.S.C. § 112, second paragraph, is moot and should be withdrawn.

With respect to claim 51, the Office alleges that claim 51 "is a method of treating limitation and therefore has no patentable weight in a pharmaceutical composition claim." (Office Action, page 5.) Without acquiescing to the rejection and solely to promote the prosecution of this case, claim 51 and its dependent claims have been canceled.

For at least the reasons set forth above, Applicants respectfully submit that the rejection under 35 U.S.C. § 112, second paragraph, should be withdrawn.

IV. The Claim Rejections Under 35 U.S.C. § 103 Should Be Withdrawn

A. The instant claims are not obvious in view of paragraph [0009]

Claims 1, 3-22, 51-55, 57, 59-61 and 72 are rejected under 35 U.S.C. § 103(a) as being allegedly unpatentable "in view of applicant's admissions at page 3, paragraph [0009], wherein applicant has admitted that 5-azacytidine is a compound well known in the art...." (Office Action, page 5.) The Office alleges that "claiming an unpatentable compound in combination with a carrier does not render the combination patentable if it would be obvious in the prior art to utilize a carrier with the compound." (Office Action, page 5.) Applicants respectfully disagree.

Contrary to what is alleged in the Office Action, the cited references, alone or in combination, do not render the claimed compositions obvious. A novel and unique element of the claimed compositions is the absence of an enteric coating, *i.e.*, a coating that is designed to

[2]Applicants respectfully note that claim 12 recites the term "anti-cancer agent," rather than "an additional therapeutic agent" as is alleged in the Office Action.

release the active ingredient beyond the acidic environment of the stomach when orally administered. In this regard, Applicants invite the Office to consider the Beach Declaration submitted herewith, which explains at length the clear prejudice in the art against oral formulations of 5-azacytidine within the claimed invention. (Beach Declaration, paragraphs 9-15) As noted in the Beach Declaration:

> At the time of the '213 application, there was a prejudice in the art against developing an oral immediate release formulation of 5-azacytidine. 5-azacytidine was associated with chemical instability in acidic environments, susceptibility to hydrolysis, enzymatic instability, and poor permeability, each of which would have dissuaded one from seeking to develop an oral immediate release formulation of 5-azacytidine. To the extent oral formulations were considered, such formulations focused on those that were protected from the acidic environment of the stomach, *e.g.*, those requiring enteric coating.
>
> (Beach Declaration, paragraph 9)

The art would have clearly taught away from formulating compositions that are not protected from the acidic environment in the stomach, *e.g.*, with an enteric coating. As discussed below, this is evidenced even by Redkar and Sands, references relied on by the Office. Thus, even if the 5-azacytidine compound itself was known at the time of the application, this is not an instance where the specifically claimed pharmaceutical composition, which is a non-enteric coated tablet, would be obvious from the prior art.

For at least the reasons set forth above, Applicants respectfully submit that the instant claims are not obvious in view of paragraph [0009].

B. The instant claims are not obvious over Redkar in view of Dintaman and Sands

Claims 1, 3-23, 31-55, 57, 59-61 and 72 are rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over Redkar *et al.* (U.S. Patent Publication No. 2006/0074046; hereinafter "Redkar"), in view of Dintaman *et al.* (*Pharmaceutical Research*, 1999, 16(10), 1550–1556; hereinafter "Dintaman") and further in view of Sands *et al.* (U.S. Patent Publication No. 2004/0162263; hereinafter "Sands"). (Office Action, at pages 6–9.)

Specifically, the Office alleges that Redkar teaches the advantages of increased solubility and stability associated with acid-salts of 5-azacytidine, and that this teaching allegedly renders obvious the oral administration of "immediate release" 5-azacytidine tablets. (Office Action at 6-9.) The Office acknowledges that Redkar does not disclose a pharmaceutical composition comprising a permeation enhancer such as the vitamin E derivative d-α-tocopheryl polyethylene glycol 1000 succinate (TPGS), as recited in claims 7-9 and 33-35, but asserts that Dintaman supplies Redkar with this disclosure. (*Id.*) With regard to Sands, the Office alleges that this reference discloses that 5-azacytidine pharmaceutical compositions may include the capability to rapidly dissolve in mildly acidic solutions. (*Id.* at 7.) The Office also asserts that Sands teaches that multiple different excipients may be combined with 5-azacytidine in the manufacture of tablets or capsules, and that the tablets may be prepared without an enteric coating. (*Id.*) The Office alleges that this alternative (*i.e.*, tablets without enteric coating) appears to be an equivalent to the immediate release solid dosage vehicle claimed in the present application. (*Id.*)

Based on these, the Examiner asserts that one skilled in the art would have been motivated to combine these references to arrive at the presently claimed pharmaceutical compositions and methods of treatment. (Office Action at 6-9.) Applicants respectfully disagree.

Redkar concerns salts of 5-azacytidine, not the free base as recited in the instant claims, and focuses on improving I.V. administration using the salts. Redkar notes that decitabine and 5-azacytidine are most typically administered by injection; however, the I.V. infusion of these compounds is limited by the decomposition of these compounds in water and the low aqueous solubility of these compounds. (Redkar at paragraph [0009].) As noted in the Beach Declaration, cold water is used to make I.V. solutions of 5-azacytidine in order to mitigate the decomposition of 5-azacytidine that would result from the use of room temperature water. However, the injection of cold 5-azacytidine fluid causes a patient pain and discomfort. (*See, e.g.*, *Id.* at paragraph [0135].) Redkar discloses that these shortcomings can be solved by using the more water stable salts of decitabine or 5-azacytidine, which allows the solid 5-azacytidine salt to be reconstituted in room-temperature fluid, and thus allows patients to be infused with this

room-temperature 5-azacytidine solution, minimizing the patient's pain. (*See, e.g., Id.* at paragraphs [0132] to [0138].)

Contrary to the Office's assertion that Redkar's teaching of the advantages of increased aqueous solubility and stability associated with the salts of 5-azacytidine appears to render obvious the presently claimed composition, there is no disclosure or suggestion in Redkar that 5-azacytidine could be exposed to stomach acid and still provide a therapeutic effect, let alone a non-enteric coated tablet of 5-azacytidine, as recited in the present claims. While Redkar contains a generic disclosure that its decitabine or 5-azacytidine salt formulation may be administered by various routes of administration, it is clear from its disclosure that the benefits of the greater stability and solubility in aqueous solutions provided by the salts of decitabine or 5-azacytidine as compared to the free base form are in relation to their utility in I.V. administration. Indeed, Redkar notes that I.V. administration is the "preferred" route of administration. (*See, e.g.*, Redkar at paragraph [177].)

As noted in the Beach Declaration:

> Redkar concerns administering specific salts of decitabine and 5-azacytidine, rather than 5-azacytidine, which is a free base, not a salt. Thus, Redkar does not concern administering 5-azacytidine, much less an oral immediate release formulation of 5-azacytidine. Instead, Redkar concerns utilizing salts of decitabine and 5-azacytidine that allegedly have greater aqueous solubility and stability than decitabine or 5-azacytidine itself, with the objective of improving IV administration, noted in paragraph [0177] as the "preferred" route of administration. For example, Redkar notes that the salt properties allow for an IV injection of a room temperature fluid (of a salt of decitabine or 5-azacytidine), avoiding the pain and discomfort associated with an IV injection of cold fluid necessitated by the "rapid decomposition" of decitabine in room temperature water. *See, e.g.*, Redkar at paragraphs [0135], [0138], and [0177].

(Beach Declaration, paragraph 10)

Furthermore, Redkar teaches away from oral formulations that are not protected from the acidic environment of the stomach. The decomposition trend taught by Redkar (5-azacytidine in cold fluid decomposes slower than in room temperature fluid) implies that 5-azacytidine would decompose to an even greater extent at body temperature. Additionally, Redkar teaches that decitabine decomposes in acidic solutions. (*See, e.g.*, Redkar at paragraph [0145].) Thus, by

teaching that the free-base (non-salt) form of cytidine analogs (like 5-azacytidine) decompose in both room-temperature (or greater) water and acid, Redkar teaches away from the instant claims that teach exposing 5-azacytidine to the warm, aqueous, and acidic environment of the stomach. This is consistent with the prejudice in the art against oral formulations noted in the Beach Declaration at paragraphs 9-15.

Dintaman is not related to compositions of 5-azacytidine or immediate-release formulations, let alone non-enteric coated tablets of 5-azacytidine. It simply reports on studies of the effect of TPGS on the inhibition of P-glycoprotein (P-gp) and concludes that co-administration of TPGS may enhance oral bioavailability of drugs due to its inhibition of P-gp in the intestine. (Dintaman at Abstract and 1555.) As noted in the Beach Declaration:

> Dintaman neither concerns 5-azacytidine nor any immediate release drug formulation, *e.g.*, one targeting release of a pharmaceutical agent in the stomach. Instead, Dintaman teaches that vitamin E TPGS is an inhibitor of P-glycoprotein (PGP), and inhibition of PGP by vitamin E TPGS in the intestine, *i.e.*, rather than the stomach, may enhance oral bioavailability of co-administered drugs. *See, e.g.*, Dintaman at abstract and page 1555.
>
> (Beach Declaration, paragraph 11)

Thus, one skilled in the art would not have been motivated to combine Dintaman with Redkar to attempt to arrive at an oral formulation that does not protect 5-azacytidine from the acidic environment of the stomach. Moreover, since Dintaman focuses on the function of TPGS in the intestine as opposed to in the stomach in relation to drug bioavailability, it teaches away from the present invention.

Sands also teaches away from the presently claimed pharmaceutical composition and the related method of treatment. Similar to Redkar, Sands teaches that cytidine analogues such as 5-azacytidine decompose in a highly acidic aqueous environment. Moreover, the compositions and methods disclosed in Sands are specifically designed to avoid the highly acidic environment of the stomach.

Sands teaches that cytidine analogues (such as 5-azacytidine) decompose in acid and water. Sands refers to cytidine analogs as "acid-labile" or "labile" in acid in the Abstract,

paragraphs [0003], [0029], [0030], [0089], [0091], and [0093], as well as claims 1, 5, 44, and 46. One skilled in the art would understand "acid-labile" as meaning "degrades in acid," and would therefore understand from Sands that 5-azacytidine would decompose in the acidic environment of the stomach. In paragraph [0012], Sands teaches that "[t]he poor bioavailability of such cytidine analogs is presumably due to the degradation of the cytidine analog by cytidine deaminases as well as their inherent chemical instability in the acidic gastric environment." In paragraph [0037], Sands teaches that "...the [cytidine analogue] is preferred not to substantially disintegrate in an acidic, aqueous medium..." In paragraph [0323], Sands teaches "[A]s decitabine is known to degrade rapidly when solvated in aqueous environment...." In paragraph [0308], Sands teaches "...the stability of decitabine in aqueous environment is much lower. It starts degrading immediately upon exposure to water. Its degradation is accelerated at acidic and basic pHs." The Beach Declaration also makes reference to paragraphs [0012] and [0037] above in describing the art's prejudice against oral formulations. (Beach Declaration, paragraph 14.)

Sands in fact teaches how to avoid exposure of an acid-labile drug to gastric juices, teaching a tablet coating that prevents release of the acid-labile drug in the stomach in order to release the drug in the small intestine. (*See*, *e.g.*, Sands at Abstract and paragraphs [0029], [0089], [0096], and [0113].) To achieve this intestine-targeted drug release, Sands describes enteric-coated tablets to shield acid-labile drugs (such as 5-azacytidine) from the highly acidic gastric environment.[3] (*See*, *e.g.*, *Id.* at paragraphs [0029] and [0030].) The enteric coating material is pH-sensitive and preferably dissolves at a threshold pH above 5.2, which is significantly higher than the pH of the gastric environment (*e.g.*, pH of about 1-2.). (*See Id.* at paragraphs [0029], [0030], [0034], [0037] [0305] and [0322] to [0326].) In view of all these teachings, one skilled in the art would understand from Sands that tablets of 5-azacytidine can be enteric-coated to avoid stomach acid-promoted decomposition. As stated in the Beach Declaration:

> Sands does not concern oral immediate release formulations of 5-azacytidine, *e.g.*, one that releases in the stomach. Instead, Sands concerns enteric coated pills of

[3] As discussed below, the instantly claimed non-enteric coated tablets were surprisingly found to possess superior properties over enteric coated tablets containing 5-azacytidine.

> decitabine or 5-azacytidine that target release in the small intestine, in order to avoid the acidic environment of the stomach. *See* Sands at abstract and paragraphs [0029],[0030], [0034],[0037],[0038],[0305], and [0322] to [0326].
>
> (Beach Declaration, paragraph 12)

The Office contends that Sands discloses that its composition is capable of rapidly dissolving in mildly acidic solutions (paragraph [0038]) and that the tablets may be prepared without an enteric coating (paragraph [0037]), an alternative embodiment that appears to be an equivalent to the claimed compositions. First, the environment in the stomach is highly acidic, rather than mild acidic. Secondly, paragraph [0037] of Sands does not disclose that the drug may be prepared without an enteric coating, as asserted in page 7 of the Office Action. Rather, it states that the pharmaceutical composition is preferred **not** to substantially disintegrate in an acidic environment. Immediately before paragraph [0037], Sands teaches that it is preferred that the drug is enteric-coated. (Sands at paragraph [0036].) A person skilled in the art would have understood that paragraph [0037] of Sands, and indeed the whole of Sands, teaches an enteric-coating that shields the composition from stomach acid and targets release of a cytidine analogue in the intestine is essential to avoid acid-promoted degradation, consistent with the conventional wisdom regarding the instability of 5-azacytidine at the time of the application. (*See, e.g., Id.* at paragraphs [0029] and [0322].)

In summary, Redkar, Dintaman and Sands, either alone or in combination, do not disclose or suggest the claimed non-enteric coated tablets. A person skilled in the art would not have had any reason or a reasonable expectation of success to combine or modify these references to attempt to arrive at the presently claimed pharmaceutical compositions or methods of treatment.

Furthermore, in view of the fact that Redkar and Sands teach cytidine analogues (like 5-azacytidine) decompose in acid, and Sands and Dintaman emphasize drug release in the intestine, these three references teach away from the present invention. As noted in the Beach Declaration:

> In sum, Redkar, Dintaman, and Sands do not concern oral immediate release compositions of 5-azacytidine as claimed in the present application. Redkar and

> Sands themselves evidence the art's clear prejudice against such compositions. Therefore, it is my opinion that one of ordinary skill in the art would not have found it obvious to pursue an oral immediate release composition of 5-azacytidine.
>
> (Beach Declaration, paragraph 15)

Thus, it is respectfully submitted that it is only through hindsight reconstruction that a skilled artisan could piece together the claimed subject matter with the cited references, as the art would have provided no reason to combine or modify the teachings of these references. *KSR Int'l Co.*, 550 U.S. at 421 (cautioning against reading the Applicant's disclosure of the claimed invention at issue into the prior art and upholding the principle of avoiding the use of hindsight reconstruction); *Panduit Corp. v. Dennison Mfg. Co.*, 810 F.2d 1561 (Fed. Cir. 1987), cert. denied, 481 U.S. 1052 (1987) (noting that using hindsight reconstruction to pick and choose among isolated disclosures in the prior art to render the claims obvious should be avoided).

For the reasons stated above, Applicants respectfully submit that claims 1 and 23, and their respective dependent claims, are patentable over Redkar in view of Dintaman and further in view of Sands. Therefore, Applicants respectfully request that the rejection under 35 U.S.C. §103(a) be withdrawn.

C. The evidence of unexpected results presented in the specification and obtained from clinical trials rebut any *prima facie* case of obviousness, as evidenced by the Beach Declaration

Even assuming, *arguendo*, that a *prima facie* case of obviousness has been established by the Office, the evidence of unexpected results presented in the specification, as well as obtained from several clinical trials presented in the Beach Declaration and its supporting exhibits, rebut any *prima facie* case of obviousness. In particular, the claimed compositions possess properties that are both significant to the treatment of MDS and surprising, particularly in view of the strong prejudice at the time of the application against pursuing the claimed compositions.

The specification presents data from studies in which three 5-azacytidine tablets were compared:

- a non-enteric coated tablet: "Formulation 6,"

- a non-enteric coated tablet containing a permeation enhancer: "Formation 3," and
- an enteric-coated tablet: "Formulation 4."

As explained in the Beach Declaration, despite the conventional wisdom at the time of the application that 5-azacytidine should not be subjected to the acidic environment of the stomach, the non-enteric coated tablets in the claimed invention exhibited superior pharmacokinetic properties compared to the enteric coated tablet. (Beach Declaration, paragraphs 17-19.) Moreover, the results surprisingly show that a permeation enhancer is not necessary to achieve the superior results. (Beach Declaration, paragraph 17.) The specific results, as summarized in the Beach Declaration in paragraphs 17-19, are as follows:

- the plasma concentration, as measured by C_{max} (the maximum concentration of an active pharmaceutical ingredient-"API"- in the blood plasma after administration) of non-enteric coated tablets of Formulations 3 and 6 is higher than the C_{max} of enteric coated tablets of Formulation 4 (see Figures 10 and 13 of the '213 application);

- the T_{max} (time to achieve C_{max}) of non-enteric coated tablets (0.5-1.0 hour) occurred approximately two hours sooner than that of enteric coated tablets (2.5-3.0 hours) (see paragraphs [0039], [0040], [0229] and [0230] and Figures 8, 9, and 10 of the '213 application);

- the non-enteric coated tablets provided a larger area under the curve ("AUC") compared to the enteric coated tablet (the AUC is the area under the curve of a concentration-of-API-in-blood-plasma vs. time plot, which is essentially a measure of the total amount of drug "seen" by the body; see Figure 12 of the '213 application); and

- the relative oral bioavailabilities of non-enteric coated Formulations 3 and 6 are higher than the relative oral bioavailability of enteric coated Formulation 4 (relative oral bioavailability is a comparison of the bioavailability of the oral formulation compared to the bioavailability of the subcutaneous formulation, *i.e.*

the ratio of the AUC of the oral form to the AUC of the subcutaneous form; see Figure 14), indicating a larger percentage of the non-enteric coated tablet (Formulation 6) is absorbed (30%) compared to the enteric coated tablet (Formulation 4; 6.7%). *See* '213 application. at paragraphs [0229] and [0230] and Figures 10 and 14.

Furthermore, as explained in paragraph 19 of the Beach Declaration, in contrast to the subcutaneous (SC) formulation, paragraphs [0225] and Figure 5 of the instant application supports that the oral immediate release formulations alter the safety profile of 5-azacytidine, permitting "the delivery of azacitidine at lower doses over a more prolonged period of time." Specifically, it was found that in a human patient study where an oral immediate release formulation of 5-azacytidine was administered starting with cycle 2, grade 3 or 4 cytopenias were not observed, yet an increase of platelets above baseline levels was still observed. This stands in contrast to SC injections administered in cycle 1, during which time grade 4 cytopenias were observed.

The data presented above would have been unexpected to those of ordinary skill in the art. Indeed, in paragraphs 17-19 of the Beach Declaration, these data are noted to be significant and surprising. Thus, Applicants respectfully submit that these data presented in the specification alone are sufficient to rebut any *prima facie* case of obviousness. However, for avoidance of any doubt, Applicants invite the Office to consider the data obtained from clinical trials (discussed in the Beach Declaration) utilizing the claimed compositions, which further support the non-obviousness of the instant claims.

In one clinical study reported in Garcia-Manero *J Clin Oncol* 29:2521, 2011 ("Garcia-Manero 1"), patients with myelodysplastic syndromes, chronic myelomonocytic leukemia, or acute myeloid leukemia (MDSs, CMML, and AML, respectively) were administered 5-azacytidine over the first 7 days of multiple 28 day cycles, and pharmacokinetic, pharmacodynamic, and clinical activity data were acquired. During the first cycle, patients received 5-azacytidine by SC injection; for all subsequent cycles patients received 5-azacytidine by oral immediate release tablets within the claimed invention. The starting oral dose was 120

mg and doses were escalated until the maximum tolerated dose (MTD) was determined to be 480 mg. (Beach Declaration, paragraph 20.)

With respect to the pharmacokinetic data, the Beach Declaration, in paragraph 21 explains:

> The pharmacokinetic data revealed the mean relative 5-azacytidine oral bioavailability ranged from 6.3% to 20%, with the bioavailability of the MTD of 480 mg having an oral bioavailability of 13% ±9%. *Id.* Pharmacokinetic data was also consistent with absorption in the stomach resulting from immediate release. *See, e.g.*, Figure 1A of Garcia-Manero 1 and Figure 10 of the '213 application.

With respect to the pharmacodynamic data, the Beach Declaration, in paragraph 22, explains:

> The pharmacodynamic results demonstrated comparable biological effects between SC injected and orally administered 5-azacytidine, specifically with respect to DNA hypomethylation. During cycles 1 and 2 of the study, DNA methylation was evaluated by counting the numbers of highly methylated loci. It was found that maximum effects presented at day 15 of each cycle. *See, e.g.*, Garcia-Manero 1 at 2526. Compared to the pre-treatment baseline, 6,981 loci were differentially methylated (6,917 hypomethylated) on day 15 of cycle 1 and 1,609 loci were differentially methylated (1,600 hypomethylated) on day 15 of cycle 2. 1,482 loci of the 1,600 loci hypomethylated by orally administered 5-azacytidine were also hypomethylated by SC injected 5-azacytidine, indicating the two routes of administration have similar biological activity. *Id.* at 2525. As noted in Garcia-Manero 1 on page 2526, "[k]inetics of the change in DNA methylation levels after SC and oral azacitidine were similar…."

The data above are both significant and surprising. The data show that the claimed compositions may allow for extended treatment schedules previously not possible with subcutaneous and intravenous administration regimens. As explained in the Beach Declaration in paragraph 23:

> In my opinion, these results are both significant and surprising, as the lower drug exposure, DNA hypomethylation results, and convenience of oral administration form the basis of an extended treatment schedule of 5-azacytidine allowing for chronic exposure to 5-azacytidine. Oral administration offers a superior alternative to IV and SC administration, which are not conducive to extended dosage schedules.

Furthermore, as explained in the Beach Declaration in paragraph 24:

> Also significant and surprising is the report in Garcia Manero 1 that clinical response was observed in 73% of first-line patients as well in 35% of previously-treated patients with MDS and CMML. Further, if the previously treated patients who showed bone marrow complete remission were included, the response rate would be 65% of previously treated patients.

The results obtained from a second clinical study reported in Garcia-Manero *Blood* Abstract 424, 2012 ("Garcia-Manero 2") and described in paragraphs 25-27 of the Beach Declaration confirm that oral azacytidine administered in extended dosing schedules is both active and well-tolerated. The study assessed the efficacy of oral 5-azacytidine in lower-risk MDS patients who were dependant on red blood cell transfusions or had thrombocytopenia (or both). (Beach Declaration, paragraph 25.) As explained in the Beach Declaration in paragraph 25, the study was performed as follows:

> Patients were split into two groups. In one group, patients were treated with 300 mg oral immediate release tablet of 5-azacytidine once a day for 14 days in repeated 28-day cycles. In another group, patients were treated with the same dose every day for 21 days in repeated 28-day cycles. 39% of patients in the 14 day cohort and 30% of patients in the 21 day cohort responded to the treatment.

As explained in the Beach Declaration in paragraph 26, the following results were obtained:

> Of the patients who received at least 4 cycles of oral 5-azacytidine, 47% of patients in the 14 day cohort and 50% of patients in the 21 day cohort responded to treatment, and 67% of the patients in the 14 day cohort and 57% of the patients in the 21 day cohort who were transfusion dependent on red blood cells gained transfusion independence. *Id.* The results of this study also indicated a positive safety profile. The most frequent hematologic adverse events included anemia (11.5% of patients), thrombocytopenia (11.5%), and neutropenia (7.7%). The most common non-hematologic adverse events were gastrointestinal and were manageable. *Id.*

The significance of the findings discussed was confirmed by Dr. Garcia Manero, who noted that the oral 5-azacytidine results are "most relevant in MDS" in a recent American Society of Hematology meeting. (Beach Declaration, paragraph 28.)

Applicants respectfully submit that the evidence of unexpected results presented in the specification and obtained from clinical trials rebut any *prima facie* case of obviousness. For at least the reasons set forth above, Applicants respectfully submit that the rejection under 35 U.S.C. § 103 should be withdrawn.

CONCLUSION

For at least the foregoing reasons, Applicants respectfully submit that all of the pending claims are in allowable form, and thus, respectfully request that the rejections be withdrawn and the application proceed to allowance. Please charge the fees for Extension of Time, and any other fees required by the Patent Office, to Jones Day Deposit Account No. 50-3013 (referencing our number 501872-999847).

Respectfully submitted,

Date	February 27, 2014	/Robert Chang/	63,753
		By: Robert Chang	Reg. No.
		For: Yeah-Sil Moon	52,042

JONES DAY
222 East 41st Street
New York, NY 10017-6702
(212) 326-3939

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: Jeffrey B. Etter *et al.*	Confirmation No.: 5370
Serial No.: 12/466,213	Art Unit: 1673
Filed: May 14, 2009	Examiner: Lawrence E. Crane
For: ORAL FORMULATIONS OF CYTIDINE ANALOGS AND METHODS OF USE THEREOF	Attorney Docket No: 9516-847-999 501872-999847

DECLARATION OF CHARLES. L. BEACH UNDER 37 C.F.R. 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Charles L. (C.L.) Beach, declare and state that:

1. I received my Bachelor of Science degree in Pharmacy from the University of Missouri-Kansas City in 1980. I received my Pharm.D. from the University of Texas Health Science Center in San Antonio, Texas in 1982.

2. From January 1983 to December 1983, I performed my residency in clinical pharmacokinetics in adult medicine at the University of Texas Health Science Center. From January 1984 to May 1984, I was a clinical instructor at the University of Texas Health Science Center. From May 1984 to August 1986, I was Coordinator of Clinical Pharmacokinetics at the University of Missouri-Columbia. From August 1986 to March 2000, I was employed at Aventis Pharmaceuticals (formerly Marion Laboratories, Marion Merrell Dow and Hoechst Marion Roussel), serving as Clinical Research Scientist (August 1986 – August 1991), Planning and Development Manager (August 1991 – March 1993), and Associate Director, Cardiovascular Medical Research (March 1993 – March 2000). From April 2000 to present, I have been employed by Celgene Corporation (first at Pharmion

Corporation prior to its acquisition by Celgene Corporation, and subsequently at Celgene Corporation), in the Hematology/Oncology division. Currently, I hold the position of Executive Director of Clinical Development.

3. I have published in peer-reviewed journals and made presentations at various academic conferences. I have over twenty-eight years of clinical research experience, including twenty-six within the pharmaceutical industry. During this time I have led implementations of clinical development and led medical research groups for both hematology and cardiovascular disease. I am responsible for VIDAZA® (5-azacytidine) clinical data and information for global regulatory submissions and approvals for high-risk MDS patients. A copy of my *curriculum vitae* is enclosed herewith as Exhibit A.

4. I have reviewed the disclosure and claims of U.S. patent application no. 12/466,213, ("the '213 application").[1] I understand that the pending claims are directed to, *inter alia*, compositions of 5-azacytidine for oral administration. I have also reviewed the Office Action issued on August 29, 2013 in connection with the '213 application. I understand that the pending claims are rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over Redkar *et al.*, (U.S. Patent Publication No. 2006/0074046; "Redkar") in view of Dintaman *et al.*, (Pharmaceutical Research, Vol. 16, No. 10, 1999; "Dintaman"), and further in view of Sands *et al.*, (U.S. Patent Publication No. 2004/0162263; "Sands"), all of which I have reviewed.

5. It is my opinion that one of ordinary skill in the art at the time the '213 application was filed would not have found it obvious to pursue an immediate-release composition of 5-azacytidine, *e.g.*, a non-enteric coated tablet, for oral administration on the basis of Redkar, Dintaman, and Sands. Furthermore, it is my opinion that immediate release oral compositions of 5-azacytidine exhibit unique

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[1] Paragraph numbers cited herein refer to those of the corresponding published application, U.S. Patent Publication No. 2009/0286752

pharmacokinetics, pharmacodynamic, and clinical properties that are both significant and surprising.

I. At the time of the '213 application, 5-azacytidine was approved for intravenous injection or subcutaneous infusion.

6. 5-azacytidine is marketed as VIDAZA®, and is the first drug approved by the FDA for the treatment of all five FAB (French-American-British) subtypes of myelodysplastic syndrome (MDS). At the time of the '213 application, the approved routes of administration were subcutaneous (SC) or intravenous (IV) injection. *See, e.g.*, VIDAZA® prescribing information, attached hereto as Exhibit B.

7. As explained in paragraph [0011] of the '213 application, the mechanism of action of 5-azacytidine is thought to involve the incorporation of 5-azacytidine into replicating DNA and RNA. In one proposed mechanism of action, the DNA-incorporated 5-azacytidine inhibits DNA-methyltransferase by forming a covalent complex between the 5-azacytosine and the enzyme. The '213 applicant explains that inhibition of DNA methyltransferases leads to DNA hypomethylation, thereby restoring normal functions to morphologically dysplastic, immature hematopoietic cells and cancer cells by re-expression of genes involved in normal cell cycle regulation, differentiation, and death.

8. The recommended injection dose of VIDAZA® is 75 mg/m^2 daily for 7 days in a 4 week cycle. IV and SC administrations of VIDAZA® are known to cause injection-site reactions including petechiae and injection site erythema. These injection site reactions, coupled with the large frequency of dosing required in the recommended treatment regimen, are burdensome to patients and negatively impact patient compliance, which in turn can prevent successful or optimal results of treatment. *See, e.g.*, the '213 application at paragraph 13; Garcia-Manero *J Clin Oncol* 29:2521, 2011 ("Garcia-Manero 1;" attached hereto as Exhibit C); and Garcia-Manero *Blood* Abstract 424, 2012 ("Garcia-Manero 2;" attached hereto as Exhibit D).

II. At the time of the '213 application, there was a prejudice in the art against developing an oral immediate release formulation of 5-azacytidine.

9. At the time of the '213 application, there was a prejudice in the art against developing an oral immediate release formulation of 5-azacytidine. 5-Azacytidine was associated with chemical instability in acidic environments, susceptibility to hydrolysis, enzymatic instability, and poor permeability, each of which would have dissuaded one from seeking to develop an oral immediate release formulation of 5-azacytidine. To the extent oral formulations were considered, such formulations focused on those that were protected from the acidic environment of the stomach, *e.g.*, those requiring enteric coating. *See, e.g.*, the '213 application at paragraph [0013].

A. Redkar, Dintaman, and Sands would not have suggested pursuing an oral immediate release composition of 5-azacytidine.

10. Redkar concerns administering specific salts of decitabine and 5-azacytidine, rather than 5-azacytidine, which is a free base, not a salt. Thus, Redkar does not concern administering 5-azacytidine, much less an oral immediate release formulation of 5-azacytidine. Instead, Redkar concerns utilizing salts of decitabine and 5-azacytidine that allegedly have greater aqueous solubility and stability than decitabine or 5-azacytidine itself, with the objective of improving IV administration, noted in paragraph [0177] as the "preferred" route of administration. For example, Redkar notes that the salt properties allow for an IV injection of a room temperature fluid (of a salt of decitabine or 5-azacytidine), avoiding the pain and discomfort associated with an IV injection of cold fluid necessitated by the "rapid decomposition" of decitabine in room temperature water. *See, e.g.*, Redkar at paragraphs [0135], [0138], and [0177].

11. Dintaman neither concerns 5-azacytidine nor any immediate release drug formulation, *e.g.*, one targeting release of a pharmaceutical agent in the stomach. Instead, Dintaman teaches that vitamin E TPGS is an inhibitor of P-glycoprotein (PGP), and inhibition of PGP by vitamin E TPGS in the intestine, *i.e.*, rather than

the stomach, may enhance oral bioavailability of co-administered drugs. *See, e.g.*, Dintaman at abstract and page 1555.

12. Sands does not concern oral immediate release formulations of 5-azacytidine, *e.g.*, on that releases in the stomach. Instead, Sands concerns enteric coated pills of decitabine or 5-azacytidine that target release in the small intestine, in order to avoid the acidic environment of the stomach. *See* Sands at abstract and paragraphs [0029], [0030], [0034], [0037], [0038], [0305], and [0322] to [0326].

B. Redkar and Sands further evidence the prejudice in the art against developing an oral immediate release formulation of 5-azacytidine.

13. Redkar and Sands further evidence the prejudice in the art against developing an oral immediate release formulation of 5-azacytidine. Redkar, at paragraph [0145], teaches that non-salt forms of decitabine and 5-azacytidine rapidly decompose in water and that decitabine decomposes "in strongly acidic solution (at pH<2.2)" to 5-azacytosine.

14. Along these lines, Sands makes reference to the art's prejudice against orally administering 5-azacytidine, noting previous reports on its "poor bioavailability" resulting from oral administration and its instability in acidic gastric environments. *See, e.g.*, Sands, at paragraph [0012]. Sands states that "[t]he poor bioavailability of such cytidine analogs is presumably due to the degradation…by cytidine deaminases as well as their inherent chemical instability in the acidic gastric environment." *Id.* Indeed, as discussed in paragraph [0037] of Sands, the compositions it discloses were formulated to "not substantially disintegrate in acidic, aqueous medium…."

15. In sum, Redkar, Dintaman, and Sands do not concern oral immediate release compositions of 5-azacytidine as claimed in the present application. Redkar and Sands themselves evidence the art's clear prejudice against such compositions. Therefore, it is my opinion that one of ordinary skill in the art would not have found it obvious to pursue an oral immediate release composition of 5-azacytidine.

III. Oral immediate release 5-azacytidine compositions disclosed in the '213 application exhibit unexpected pharmacokinetic, pharmacodynamic, and clinical properties.

A. The '213 application

16. The '213 application discloses data from studies in which two versions of non-enteric coated tablets (immediate release tablets; Formulations 3 and 6, or "F3" and "F6") were compared to an enteric coated tablet formulated to dissolve at a pH greater than 5 (delayed release tablet designed to dissolve in the lower intestine; Formulation 4, or "F4"). *See, e.g.*, the '213 application at paragraphs 209 to 243. To ensure direct comparison, each of these three tablets contained 60 mg of 5-azacytidine. The two non-enteric coated tablets were differentiated by the presence of vitamin E TPGS as a permeation enhancer (Formulation 3) and absence of vitamin E TPGS (Formulation 6). *See, e.g.*, the '213 application at Table 4.

17. The '213 application discloses that non-enteric coated tablets of 5-azacytidine, *i.e.*, tablets that do not have a coating that protects 5-azacytidine from exposure to gastric juices, have superior pharmacokinetic properties as compared to enteric coated tablets, even in the absence of a permeation enhancer. Specifically, the '213 application reports the following significant findings:

- the plasma concentration, as measured by C_{max} (the maximum concentration of an active pharmaceutical ingredient-"API"- in the blood plasma after administration) of non-enteric coated tablets of Formulations 3 and 6 is higher than the C_{max} of enteric coated tablets of Formulation 4 (see Figures 10 and 13 of the '213 application);

- the T_{max} (time to achieve C_{max}) of non-enteric coated tablets (0.5-1.0 hour) occurred approximately two hours sooner than that of enteric coated tablets (2.5-3.0 hours) (see paragraphs [0039], [0040], [0229] and [0230] and Figures 8, 9, and 10 of the '213 application);

- the non-enteric coated tablets provided a larger area under the curve ("AUC") compared to the enteric coated tablet (the AUC is the area under the curve of a concentration-of-API-in-blood-plasma vs. time plot – it essentially provides the total amount of drug "seen" by the body; see Figure 12 of the '213 application); and

- the relative oral bioavailabilities of non-enteric coated Formulations 3 and 6 are also higher than the relative oral bioavailability of enteric coated Formulation 4 (relative oral bioavailability is a comparison of the bioavailability of the oral formulation compared to the bioavailability of the SC formulation, *i.e.* the ratio of the AUC of the oral form to the AUC of the SC form; see Figure 14). Thus, a larger percentage of the non-enteric coated tablet (Formulation 6) is absorbed (30%) compared to the enteric coated tablet (Formulation 4; 6.7%). *See* '213 application. at paragraphs [0229] and [0230] and Figures 10 and 14.

18. In my opinion, these results are significant and surprising, particularly given the conventional wisdom at the time of the '213 application that 5-azacytidine would decompose in the acidic environment of the stomach.

19. The '213 application also reports in paragraph [0225] and Figure 5 that in a human patient study where an oral immediate release formulation of 5-azacytidine was administered starting with cycle 2, grade 3 or 4 cytopenias were not observed, yet an increase of platelets above baseline levels was still observed. This stands in contrast to SC injections administered in cycle 1, during which time grade 4 cytopenias were observed. The '213 application notes that this data supports that the oral immediate release formulations alter the safety profile of 5-azacytidine and "permit the delivery of azacitidine at lower doses over a more prolonged period of time…."

B. Clinical trials of oral 5-azacytidine

20. The results of a phase I dose-escalation trial reported in Garcia-Manero 1 confirmed the clinical significance of the results discussed above. In this study, patients with myelodysplastic syndromes, chronic myelomonocytic leukemia, or acute myeloid

leukemia (MDSs, CMML, and AML, respectively) were administered 5-azacytidine over the first 7 days of multiple 28 day cycles, and pharmacokinetic, pharmacodynamic, and clinical activity data were acquired. During the first cycle, patients received 5-azacytidine by SC injection; for all subsequent cycles patients received 5-azacytidine by oral immediate release tablets. The starting oral dose was 120 mg and doses were escalated until the maximum tolerated dose (MTD) was determined to be 480 mg. *Id.* at 2524.

21. The pharmacokinetic data revealed the mean relative 5-azacytidine oral bioavailability ranged from 6.3% to 20%, with the bioavailability of the MTD of 480 mg having an oral bioavailability of 13% ±9%. *Id.* Pharmacokinetic data was also consistent with absorption in the stomach resulting from immediate release. *See, e.g.*, Figure 1A of Garcia-Manero 1 and Figure 10 of the '213 application.

22. The pharmacodynamic results demonstrated comparable biological effects between SC injected and orally administered 5-azacytidine, specifically with respect to DNA hypomethylation. During cycles 1 and 2 of the study, DNA methylation was evaluated by counting the numbers of highly methylated loci. It was found that maximum effects presented at day 15 of each cycle. *See, e.g.*, Garcia-Manero 1 at 2526. Compared to the pre-treatment baseline, 6,981 loci were differentially methylated (6,917 hypomethylated) on day 15 of cycle 1 and 1,609 loci were differentially methylated (1,600 hypomethylated) on day 15 of cycle 2. 1,482 loci of the 1,600 loci hypomethylated by orally administered 5-azacytidine were also hypomethylated by SC injected 5-azacytidine, indicating the two routes of administration have similar biological activity. *Id.* at 2525. As noted in Garcia-Manero 1 on page 2526, "[k]inetics of the change in DNA methylation levels after SC and oral azacitidine were similar…."

23. In my opinion, these results are both significant and surprising, as the lower drug exposure, DNA hypomethylation results, and convenience of oral administration form the basis of an extended treatment schedule of 5-azacytidine allowing for chronic exposure to 5-azacytidine. Oral administration offers a superior alternative

to IV and SC administration, which are not conducive to extended dosage schedules. As noted in Garcia-Manero 1 on page 2527, such extended and continued administration of 5-azacytidine would likely positively affect the clinical activity of 5-azacytidine.

24. Also significant and surprising is the report in Garcia Manero 1 that a clinical response was observed in 73% of first-line patients as well in 35% of previously-treated patients with MDS and CMML. Further, if the previously treated patients who showed bone marrow complete remission were included, the response rate would be 65% of previously treated patients. *Id.* at 2526.

25. In another clinical study, reported in Garcia-Manero 2, the efficacy of oral 5-azacytidine in lower-risk MDS patients who were dependant on red blood cell transfusions or had thrombocytopenia (or both) was assessed. Patients were split into two groups. In one group, patients were treated with 300 mg oral immediate release tablet of 5-azacytidine once a day for 14 days in repeated 28-day cycles. In another group, patients were treated with the same dose every day for 21 days in repeated 28-day cycles. 39% of patients in the 14 day cohort and 30% of patients in the 21 day cohort responded to the treatment. *See, e.g.*, Garcia-Manero 2.

26. Of the patients who received at least 4 cycles of oral 5-azacytidine, 47% of patients in the 14 day cohort and 50% of patients in the 21 day cohort responded to treatment, and 67% of the patients in the 14 day cohort and 57% of the patients in the 21 day cohort who were transfusion dependent on red blood cells gained transfusion independence. *Id.* The results of this study also indicated a positive safety profile. The most frequent hematologic adverse events included anemia (11.5% of patients), thrombocytopenia (11.5%), and neutropenia (7.7%). The most common non-hematologic adverse events were gastrointestinal and were manageable. *Id.*

27. In my opinion, these results are both significant and surprising, as they confirm that immediate release tablet of 5-azacytidine orally administered in extended dosing schedules is both active and well-tolerated.

28. Recently, at the American Society of Hematology (ASH) meeting in December 2013, Dr. Garcia-Manero discussed his excitement over oral immediate release tablet of 5-azacytidine, noting that the oral 5-azacytidine results are "most relevant in MDS." He was particularly excited by the prospect of oral 5-azacytidine to treat patients who did not respond to previous treatment, as there is currently no approved second-line pharmaceutical agent for this population. *See, e.g.*, http://www.youtube.com/watch?v=QyV8P_DoLTc&feature=em-share_video_user

29. I, Charles L. Beach, declare that all statements made herein are of my own knowledge to be true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent that may issue there from.

Dated: 25 FEB 2014

C. A. Beach

Charles L. Beach

EXHIBIT A

Charles L. (C.L.) Beach, Pharm.D., B.S. Pharm.

Celgene Corporation
Suite 900
9225 Indian Creek Parkway
Overland Park, KS 66210

Office Phone: 913-266-0302
Cell Phone: 816-694-2926
e-mail: clbeach@celgene.com

PROFESSIONAL EXPERIENCE: Twenty-eight years of clinical research experience, 26 within the pharmaceutical industry. Twelve years experience in hematology/oncology clinical research and drug development, 12.5 years in cardiovascular clinical research and drug development and 1.5 years in cardiovascular medical education and professional education. Successful conduct of global Phase I-IV clinical trials leading to FDA, EU, Australian, Canadian and Asian regulatory approvals of Vidaza® for the treatment of and prolonging survival in Myelodysplastic Syndromes (MDS) and Cardizem CD for the treatment of Angina Pectoris in the U.S and Australia. Activities have included clinical development strategy, regulatory strategy, clinical study development, clinical study conduct, data analysis, report writing, publishing and US and International regulatory submissions and defense.

Led implementations of clinical development and medical research groups for hematology within Celgene/Pharmion and for cardiovascular within Hoechst Marion Roussel.

Extensive experience and interactions with national and international thought leaders for hematology and cardiovascular medicine. These interactions led to clinical program development strategy, regulatory submission strategy, regulatory responses strategy and professional education strategy for the US, Canada, EU, Australia, South America, and Asian countries. Extensive interaction with patient advocacy groups and national organizations these have included the NKF, NIDDK, MDS foundation, and NCI.

This experience is inclusive of 19 years of extensive interaction with marketing and commercial organization for the approval of hematology and cardiovascular sales information, sales representative training and educational materials.

Celgene Corporation (formerly Pharmion Corp.)

April 2000 to Present — **Executive Director Clinical Development, Hematology/Oncology**

Activities include the following:

Clinical lead responsible for the development of Vidaza clinical data and information for global regulatory submissions and approvals (including a survival benefit) for high risk MDS patients.

US and global clinical trials strategy and clinical trial development for myelodysplastic syndromes and elderly acute leukemia.

Development of clinical trial strategy and Phase III research trial for Vidaza in elderly acute myelogenous leukemia, being conducted internationally in 18 countries.

Development of clinical trial for assessment of Vidaza treatment in high risk MDS for Asian patients.

Development of clinical strategy and clinical trial assessment for:

- Oral Azacitidine for low risk MDS, AML post induction chemotherapy maintenance and post bone marrow transplant maintenance
- Vidaza in combination with Revlimid in elderly AML
- Vidaza in pediatric patients with MDS and AML

Assessment and approval of investigator initiated clinical trials for MDS and AML for Vidaza and Revlimid to meet the overall global development strategy.

Scientific Experieince:

- Development and implemention of Vidaza for elderly AML, a global regiatration trial of 480 patients conducted in 18 countries.
- Develop, implement and complete randomized phase III international survival trial of Vidaza for high risk MDS patients. Inclusive of data analysis review, report writing, defense of regulatory submissions and publication of data.
- Development of strategy and data presentation to the Taiwanese regulatory agency for approval of Vidaza for MDS.
- Development and implementation of a Phase IV trial for Vidaza in high risk MDS Taiwanese patients.
- Development of clinical strategy for Vidaza data presentation to Chinese regulatory authorities.
- Development and implementation of a Phase II trial for Vidaza in high risk MDS Chinese patients.
- Strategy development for additional data analysis and publication of Vidaza survival dataset. This is inclusive of traditional clinical endpoints and epigenetic markers for disease diagnosis and therapeutic interventions.
- Developed, implemented and published a randomized alternative drug administration schedule trial for Vidaza in MDS patients to avoid administration schedule difficulties.
- Developed, implemented, completed and published Phase I PK trial for evaluation of intravenous to subcutaneous Vidaza.
- Developed and implemented strategy for modeling of pharmacokinetic Vidaza IV/SC PK data leading to FDA submission and approval for updated US labeling.
- Global Investigator Initiated Trials; contribute to strategy development, review and approve all trials.
- Evaluate the development possibilities for demethylation agents in the various solid tumors and solid tumor models. These include ovarian, prostate, lung and breast.
- Develop strategy for Vidaza in pediatric patients to meet criteria for FDA pediatric research legislation. Phase I/II pharmacokinetic and efficacy evaluation of injectable Vidaza in pediatric patients with liquid and solid tumors failing previous therapy.
- Clinical strategy for Vidaza in pediatric patients to meet criteria for European pediatric research legislation. Phase I/II pharmacokinetic and efficacy evaluation of injectable Vidaza in pediatric patients with hematoligic malignancy.
- Led team to recollect and reanalyze MDS data from 3 CALGB MDS trials (312 patients at 84 study sites) for FDA submission and approval of MDS.
- Develop and implement strategy for the use of DNA metyltrasferase inhibitor use alone and in combination with histone deacetylation inhibitors for the treatment of high risk MDS and AML patients.
- Develop strategy for analysis of CALGB MDS clinical response data to a modern classifications system (International Working Group for MDS 2000) for comparative analysis.
- Authored clinical study reports and clinical/efficacy sections of NDA's for US, EU, Australia, Canada and Asia for MDS submissions.
- Multiple interactions with national and international thought leaders to develop clinical strategy for high risk MDS and AML.
- Experienced in the development and management of therapeutic advisory boards, national and international investigator meetings, centralized hematopathology and cytogenetic study processes, and centralized event adjudication.
- Provide review and evaluation of Vidaza protocols submitted to the NCI (CTEP) for funding.
- Clinical lead for pharmacoeconomic data analysis and modeling of Vidaza survival data for European launch needs.
- Provide strategic input for development of oral Vidaza formulation for MDS.
- Review and approve Statistical analysis plans for Pharmion sponsored clinical trials.
- Clinical evaluation for new products (2000-2003).
  - Led due diligence team for candidate products.

- Review cardiovascular products with the Pharmion team. This included product data review provided by the sponsor, independent data acquisition, existing medical literature evaluation, and discussion with clinicians/scientists. Additional therapeutic areas included diabetes, iron chelating and echolocation.
- Develop Pharmion relationships with clinicians and other companies in the areas of cardiology, invasive radiology, vascular surgery and anticoagulation specialists.

Regulatory Experience:

- Lead clinical team for clinical responses to global regulatory submissions and subsequent question for Vidaza.
- Led Clinical team for scientific advice for pediatric development of Vidaza with the EMA
- Led Clinical team for scientific advice for treatment of elderly AML with Vidaza with the EMA
- Led NDA clinical team for filing of NDA for Vidaza injection for the treatment of MDS in 2003 and EMEA in 2004.
- Clinical lead for global regulatory submissions. Authored and approved clinical, overview and summary sections of US and EMA regulatory submissions. Authored and reviewed annual IND updates and investigator brochures.
- FDA interaction: Negotiated to gain consensus on clinical pharmacokinetic plan, pre-clinical activity, pivotal clinical survival trial and Special Protocol Assessment.
- Successfully supported Orphan application for AML in the EU and pending approval in the US.
- Prepared clinical sections and rationale for pediatric study request per FDA pediatric exclusivity legislation.
- Provided response to clinical data questions for international regulatory submissions South Korea, South Africa and Hong Kong.
- Led efforts for successful pharmacokinetic modeling of Vidaza bioavailability data for FDA label extension for IV drug administration.

Clinical Operation Experience:

- Managed operations group for two years, hired clinical operations associates during the development of Pharmion's Clinical operations group.
- Ran MDS trials in the U.S., U.K., Western and Eastern Europe and Australia at >150 centers
- Evaluated RFP's, negotiated contracts and provided oversight for the management of CRO's (PPD, PRA and Quintiles) for US, international trials for MDS survival, clinical and pharmacokinetic trials.

Management Experience:

- Led Vidaza FDA clinical submission team
- Led Clinical Project Team.
- Prepare and manage clinical budget.
- Led due diligence team for candidate products.

Additional Activities:

- Medical Science Liaisons training for MDS.
- European Medical Director training for MDS.
- Provide criteria for selection of clinical investigators for the US and EU to meet scientific and commercial need for product development.
- Develop strategy for additional analysis, publications and presentations for Vidaza Survival data
- Review and approve clinical research SOP's.
- Develop initial Medical Informatics relationship and association for Pharmion.

Marketing/Commercial:

- Medical review and approval of all marketing/commercial materials for sales distribution, selling material, education and training materials for physician, pharmacy, nursing and patients distribution.
- Medical review and approval of sales force education and training material for MDS, AML, and epigenetics for Vidaza.

Aventis Pharmaceuticals: formerly Marion Laboratories, Marion Merrell Dow and Hoechst Marion Roussel.

Employment Duration **August 1986 to March 2000**

March 1993 to March 2000 **Associate Director, Cardiovascular Medical Research (Phase 3B-4)**

Responsibilities:

- Responsible for the development and implementation of the medical operating plan for Cardizem® (diltiazem hydrochloride) for the treatment of hypertension, angina pectoris and acute heart rate control for atrial fibrillation and flutter. Additional product responsibility included Altace® and Teczem® for hypertension and Refludan® for heparin-induced thrombocytopenia.
- Hired and trained 2 Medical Research Scientists
- Development and oversight of medical research protocols, investigator IND's, IND exempt protocols, supported National Institute of Health programs, and supported Veterans Administrations Cooperative programs.
- Led team for development of Clinical Effectiveness Trials program.
- Led the integration of Health Economic and Pharmacoeconomic activity into medical research post marketing cardiovascular programs.
- Cardiovascular Disease State Management development with health care maintenance organizations.
- IND and NDA maintenance for Cardizem tablets, Cardizem SR (BID formulation), Cardizem CD (once daily formulation, Cardizem IV (intravenous) and Altace.
- Support FDA responses for marketed cardiovascular products.
- Led the program for defense calcium channel blocks induced myocardial infarction during the treatment of hypertension.
- Medical review and approval of all sales and marketing promotional materials and sales training materials with cardiovascular information. Products include Cardizem family, Teczem, Altace and Refludan (lepirudin).
- Author internal medical research reports.
- Package inserts update and development.
- Educate Medical Science Liaisons in the selection of clinical study investigator selection and submission of investigator initiated trial proposals

Key Activities:

- Led team for development of Clinical Effectiveness Trial program for Medical Research. Inclusive of a US based study of 1700 sites and 5400 patients for the treatment of hypertension with Altace vs. Losartan.
- Led the program for defense calcium channel blocks induced myocardial infarction during the treatment of hypertension. This included review of Cardizem clinical data with evaluation by academic experts in hypertension, ischemic heart disease and diabetes. Led the development of a definitive trial treating hypertension patients for the development of myocardial infarctions using Cardizem CD vs. conventional hypertension treatment with a diuretic. In consultations with a group of US key hypertension and cardiovascular therapeutic advisors we developed an 8400 patient trial. The trial was at the state of initiation with clinical study sites selected and CRO contracted when the company decided to not proceed with the trial.

August 1991 to March 1993 **Planning and Development Manager**
Cardiovascular Medicine
Professional Education and Scientific Communications

Responsibilities:

- Development and implantation of cardiovascular education and scientific presentations for pre and post-market cardiovascular products (primary product Cardizem®).

- Develop symposia and educational programs for physicians (cardiologists, nephrologists, electrophysiologists, hypertensionlogists, internal medicine, and primary care physicians) and additional health care professionals (Clinical pharmacists, pharmacists, nurses, and nurse practioners).
- Develop/support and implement regional and national visiting speaker programs.
- Develop/support and implement educational programs for cardiology fellows and chief medical residents.
- Advocate development.

August 1986 to August 1991 — **Clinical Research Scientist**
Cardiovascular Medicine

Responsibilities:

- Therapeutic area- myocardial ischemia working with the calcium channel blocker Cardizem.
- Design and implemented multi-center antianginal programs for a twice daily Cardizem product to support an NDA supplement.
- Design and implemented multi-center antianginal programs for a once daily Cardizem product to support an NDA supplement.
- Design and implemented a program for acute (unstable) myocardial ischemia with Cardizem.
- Design a program for assessing acute effects of intravenous Cardizem on myocardial systolic and diastolic function.
- Author clinical research reports and clinical research sections for NDA antianginal supplements.
- Review clinical data for licensing of potential cardiovascular products.

University of Missouri- Columbia

May 1984 to August 1986 — **Coordinator of Clinical Pharmacokinetics**
University of Missouri-Columbia
Hospitals and Clinics

Responsibilities:

- Developed inpatient pharmacokinetic consult services.
- Conduct cardiovascular clinical research in conjunction the department of cardiology.
- Provide clinical pharmacy service in acute cardiology.
- Participate in the Pharmacy and Therapeutic drug selection committee for the University Hospitals and Clinics.

University of Texas Heath Science Center- San Antonio

January 1984 to May 1984 — **Clinical Instructor**
Clinical Pharmacy Program

Responsibilities:

- Provide clinical instruction and evaluation of PharmD residents in the adult cardiology and clinical pharmacokinetics.

POST GRADUATE TRAINING:

January 1983 to December 1983 — Residency
Clinical Pharmacokinetics in Adult Medicine
(Adult/Acute Cardiology)
University of Texas Clinical Pharmacy Program
University of Texas Health Science Center
San Antonio Texas and the Audie Murphy Memorial Veterans Hospital, San Antonio, Texas.

EDUCATION:

July 1980 to December 1982	Doctor of Pharmacy University of Texas Clinical Pharmacy Program University of Texas Health Science Center San Antonio, Texas
August 1977 to July 1980	Bachelor of Science in Pharmacy University of Missouri-Kansas City School of Pharmacy

ORAGANIZATION MEMBERSHIP:

American Society of Hematology
American Society of Clinical Oncology

REFERENCES:

United States

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Australia

Associate Prof. John Seymour, MB, MS, PhD, FRACP, Peter MacCallum Cancer Centre, Head, Haematology Department & Chair, Haematology Service, Division of Haematology/Medical Oncology 5th Floor, 7 St. Andrew's Place, East Melbourne, VIC 3002, Australia, 61 3 9656 1076, john.seymour@petermac.org

PUBLICATIONS:

Manuscripts:

1. Gidwani R. Kahan ZM, Fenaux P, **Beach CL,** Pashos CL. A cost-effective analysis of using **azacitidine** vs. decitabine in treating patients with myelodysplastic syndromes. J Med Econ.2012;15:145-154.

2. Prébet T, Gore SD, Esterni B, Gardin C, Itzykson R, Thépot S, Dreyfus F, Rauzy OB, Recher C, Adès L, Quesnel B, **Beach CL,** Fenaux P, Vey N. Outcome of high-risk myelodysplastic syndrome after **azacitidine** treatment failure. J Clin Oncol. 2011 Aug 20;29(24):3322-7. Epub 2011 Jul 25.

3. Itzykson R, Thépot S, Quesnel B, Dreyfus F, Beyne-Rauzy O, Turlure P, Vey N, Recher C, Dartigeas C, Legros L, Delaunay J, Salanoubat C, Visanica S, Stamatoullas A, Isnard F, Marfaing-Koka A, de Botton S, Chelghoum Y, Taksin AL, Plantier I, Ame S, Boehrer S, Gardin C, **Beach CL,** Adès L, Fenaux P; Groupe Francophone des Myelodysplasies(GFM). Prognostic factors for response and overall survival in 282 patients with higher-risk myelodysplastic syndromes treated with **azacitidine.** Blood. 2011 Jan 12:117(2):403-11. Epub 2010 Oct 12.

4. Seymour JF, Fenaux P, Silverman LR, Mufti GJ, Hellström-Lindberg E, Santini V. List AF, Gore SD, Backstrom J, McKenzie D. **Beach CL.** Effects of **azacitidine** compared with conventional care regimens in elderly (≥75 years) patients with higher-risk myelodysplastic syndromes. Crit Rev Oncol Hematol. 2010 Dec;76(3):218-27. Epub 2010 May 6.

5. Santini V, Fenaux P, Mufti GJ, Hellström-Lindberg E, Silverman LR, List A, Gore SD, Seymour JF, Backstrom J. **Beach CL.** Management and support care measures for adverse events in patients with myelodysplastic syndromes treated with **azacitidine*.** Eur J Haematol. 2010 Aug;85(2):130-8. Epub 2010 Apr 12.

6. Fenaux P, Gattermann N. Seymour JF, Hellström-Lindberg E, Mufti GJ, Duehrsen U. Gore SD, Ramos F, Beyne-Rauzy O, List A, McKenzie D, Backstrom J, **Beach CL**. Prolonged survival with improved tolerability in higher-risk myelodysplastic syndromes: **azacitidine** compared with low dose ara-C. Br J Haematol. 2010 Apr;149(2):244-9. Epub 2010 Feb 5. Erratum in: Br J Haematol. 2010 Jun; 149(6):919.

7. Fenaux P. Mufti GJ, Hellström-Lindberg E. Santini V, Gattermann N. Germing N, Germing U, Sanz G. List AF, Gore S, Seymour JF, Dombret H, Backstrom J, Zimmerman L, McKenzie D, **Beach CL,** Silverman LR. **Azacitidine** prolongs overall survival compared with conventional care regimens in elderly patients with low bone marrow blast count acute myeloid leukemia. J Clin Oncol. 2010 Feb 1;28(4):562-9. Epub 2009 Dec 21.

8. Lyons RM, Cosgriff TM, Modi SS, Gersh RH, Hainsworth JF, Cohn AL, McIntyre HJ, Fernando IJ, Backstrom JT, **Beach CL.** Hematologic response to three alternative dosing schedules of **azacitidine** in patients with myelodyspolatic syndromes. J Clin Oncol. 2009 Apr 10;27(11):1850-6. Epub 2009 Mar 2.

9. Fenaux P. Mufti GJ, Hellström-Lindberg E, Santini V, Finelli C, Giafounidis A, Schoch R, Gattermann N, Sanz G. List A, Gore SD, Seymour JF, Bennett JM, Byrd J, Backstrom J, Zimmerman L, McKenzi D, **Beach CL,** Silverman LR: International Vidaza High-Risk MDS Survival Study Group. Efficacy of **azacitidine** compared with conventional care regimens in the treatment of higher-risk myelodysplastic syndromes: a randomized, open-label, phase III study. Lancot Oncology 2009:10(3):223-32.

10. Silverman LR, McKenzie D R, Peterson B L, Holland J F, Backstrom J T, **Beach CL**, Larson R A. Further analysis of trials with **azacitidine** in patients with myelodysplastic syndrome: studies 8421, 8921, and 9221 by the cancer and leukemia group B. Journal of Clinical Oncology. 2006 Aug 20;24(24):3895-3903

11. Marcucci G, Silverman L, Eller M, Lintz L, **Beach CL**. Bioavailability of **azacitidine** subcutaneousversus intravenous in patients with the myelodysplastic syndromes. J Clin Pharmacol. 2005;45(5):597-602.

12. Herrera CR, Lewin A, Fiddes R, Friedman J, Linn W, Baker T, Balanoff D, **Beach CL**. Long-Acting Diltiazem CD Is Safe and Effective in a Hypertensive Mexican-American Population. Pharmacotherapy 1997, 17: 1254-1259.

13. Heller GV, Sridharan M, Morse J, Glassar S, **Beach CL**. Antianginal Response To Once-Daily Diltiazem CD in Patients Receiving Concomitant Beta-Blockers, Long-Acting Nitrates, or Both. Pharmaoctherapy 1997, 17:760-766.

14. Blackshear JL, Stambler BS, Strauss WE, Roy D, Dias VC, **Beach CL**, Ebener MR. Control of Heart Rate During Transition From IV to Oral Diltiazem in Patients With Atrial Fibrillation or Flutter. Am J Cardiology 1996, 78:1246-1250.

15. Thadani U, Glassar S, Bittar N, **Beach C**. Dose Response Evaluation of Once-Daily Therapy With A New Formulation of Diltiazem For Stable Angina Pectoris. Am J Cardiology 1994, 74:9-17.

16. Alpert MO, Mukerji V, Villarreal P,Singh A, Flaker GC, Sanfelippo JF, **Beach CL**, Morgan RJ. Efficacy of Betaxolol in the Treatment of Stable Exertional Angina Pectoris: A Dose- Ranging Study. Angiology 1990, 41:365-376.
17. **Beach CL**, Farringer JA, Peck CC, Crawford MH, et al. Clinical Assessment of a Two-Compartment Bayesian Forecasting Method for Lidocaine. Therapeutic Drug Monitoring 1988; 10:74-79.
18. Flaker GC, **Beach CL**, Chapman D. Adverse Side Effects Associated with Mexiletine. Clinical Progress in Electrophysiology and Pacing 1986; 4:602-607.
19. **Beach CL**, Ludden TM, Clementi WA, Allerheiligen SR. Measurements of Lidocaine Free Concentrations. Therapeutic Drug Monitoring 1986; 8:326-330.
17. Mukerji V, Alpert MA, Flaker GC, **Beach CL**, Weber RD. Cardiac Conduction Abnormalities and Atrial Arrhythmias Associated with Salicylate Toxicity. Pharmacotherapy 1986; 6:41-43.
18. **Beach CL**. Theophylline/Phenytoin Interaction. Drugdex, 24 edition, 1984.
19. **Beach CL**. Cimetidine/Theophylline Interaction. Time course for reversal. Drugdex, 22 edition, 1984.

Abstracts:

ADD ASH 2012 ABSTRACTS…..

1. Tao Shi, Ludmila Danilova, Xiaolong Luo, Lela M. Lucy, **CL Beach**, Leslie Cope, James G. Herman, Kyle J. MacBeth. **Identification of a DNA Methylation Predictive Signature of Overall Survival in Higher-Risk MDS Patients Treated with Azacitidine.** *Blood* (ASH Annual Meeting Abstracts) 2011; 118: abstract 3815.
2. Eric Laille, Alain C. Mita, Sanjay Goel, Nashat Y. Gabrail, Joseph Schwartz, **CL Beach**. **A 2-Part Phase I Study in Patients with Solid or Hematologic Malignancies: Dose Proportionality of Subcutaneous (SC) Azacitidine (AZA) and Pharmacokinetics of SC AZA in Patients with Severe Renal Impairment**, *Blood* (ASH Annual Meeting Abstracts) 2011; 118; 3480.
3. John Koreth, Joseph Pidala, Waleska S. Perez, H. Joachim Deeg, Guillermo Garcia-Manero, Luca Malcovati, Mario Cazzola, Sophie Park, Raphael Itzykson, Lionel Ades, Pierre Fenaux, Martin Jädersten, Eva Hellström-Lindberg, Robert Peter Gale, **C.L. Beach, PharmD**, Peter L. Greenberg, Martin S. Tallman,, John F. DiPersio, Donald Bunjes, Daniel J. Weisdorf, Corey S. Cutler, **A Decision Analysis of Reduced-Intensity Conditioning Allogeneic Hematopoietic Stem Cell Transplantation for Older Patients with De-Novo Myelodysplastic Syndrome (MDS): Early Transplantation Offers Survival Benefit in Higher-Risk MDS.** *Blood* (ASH Annual Meeting Abstracts) 2011 118: Abstract 115
4. S. Gore, P. Fenaux, V. Santini, J. M. Bennett, L. R. Silverman, J. F. Seymour, E. Hellstrom-Lindberg, A. S. Swern, **C.L. Beach**, A. F. List. **Time-dependent decision analysis: Stable disease in azacitidine (AZA)-treated patients (pts) with higher-risk MDS.** *J Clin Oncol* 28:15s, 2010 (suppl; abstr 6503)
5. Raphael Itzykson, Sylvain Thepot, Bruno Quesnel, Francois Dreyfus, Odile Beyne-Rauzy, Pascal Turlure, Christian Recher, Caroline Dartigeas, Norbert Vey, Laurence Legros, Jacques Delaunay, Celia Salanoubat, Sorin Visanica, Aspasia Stamatoullas, Françoise Isnard, Anne Marfaing-Koka, Stephane De Botton, Youcef Chelgoum, Anne-Laure Taksin, Isabelle Plantier, Shanti Ame, Simone Boehrer, Claude Gardin, **CL Beach**, Lionel Ades, and Pierre Fenaux. **A Prognostic Score for Overall Survival (OS) with Azacitidine (AZA) In Higher Risk MDS Based on 282 Patients (pts), and Validated In 175 Pts From the AZA 001 Trial**
   Blood (ASH 2010 Annual Meeting Abstracts) 116: 3996.
6. Thomas Prebet, Zhuoxin Sun, Rhett Ketterling, Gary Hicks, **C.L. Beach**, Peter L. Greenberg, Elisabeth Paietta, Magdalena Czader, Janice Gabrilove, Harry P Erba, Martin S. Tallman, and Steven D. Gore. **A 10 Day Schedule of Azacitidine Induces More Complete Cytogenetic Remissions Than the Standard Schedule In Myelodysplasia and Acute Myeloid Leukemia with Myelodysplasia-Related Changes: Results of the E1905 US Leukemia Intergroup Study**
   Blood (ASH 2010 Annual Meeting Abstracts) 116: 4013.

7. Norbert Gattermann, Guillermo F. Sanz, Aristoteles Giagounidis, John F Seymour, Pierre Fenaux, Valeria Santini, Ghulam J. Mufti, Petra Muus, Fernando Ramos, Lela M. Lucy, **CL Beach**, and Lewis R. Silverman **Re-Evaluation of the Efficacy of Azacitidine (AZA) In Patients From the AZA-001 Study with Higher-Risk Myelodysplastic Syndromes (MDS) Classified by WHO Criteria and WPSS Risk** *Blood* (ASH 2010Annual Meeting Abstracts) 116: 4031.

8. Thomas Prebet, Steven D. Gore, Benjamin Esterni, Raphael Itzykson, Sylvain Thepot, **C.L. Beach**, Pierre Fenaux, and Norbert Vey. **Outcome of Patients (pts) Treated for Myelodysplastic Syndrome (MDS) and Secondary Acute Myeloid Leukemia (s AML) After Azacitidine (AZA) Failure** *Blood* (ASH 2010Annual Meeting Abstracts) 116: 443.

9. John F Seymour, Valeria Santini, Pierre Fenaux, Aristoteles A.N. Giagounidis, Guillermo F. Sanz, Carlo Finelli, Lela M. Lucy, Jay Backstrom, and **CL Beach. Achievement of Red Blood Cell (RBC) Transfusion Independence with Azacitidine (AZA) Leads to Improved Survival In Patients with Higher-Risk Myelodysplasias Regardless of Baseline Transfusion Needs** *Blood* (ASH 2010 Annual Meeting Abstracts) 116: 1856.

10. Raphael Itzykson, Sylvain Thepot, Odile Beyne-Rauzy, Bruno Quesnel, Shanti Ame, Pascal Turlure, Stephanie Harel, Anne Marfaing-Koka, Youcef Chelgoum, Aspasia Stamatoullas, Madalina Uzunov, Norbert Ifrah, Herve Dombret, Lionel Ades, **CL Beach**, Arlene S. Swern, Claude Gardin, and Pierre Fenaux **Prolonged Survival without Complete Remission (CR) In AML Patients (PTS) Treated with Azacitidine (AZA)** *Blood* (ASH 2010 Annual Meeting Abstracts) 116: 2183.

11. J. Herman ,G.J. Mufti ,K. J. MacBeth ,J. F. Seymour ,P. Fenaux ,S.D. Gore ,V. Santini ,L. M. Lucy ,**C.L. Beach. An Evaluation of the Association of CDKN2B (P15) DNA Methylation with response and survival in patients with higher-risk myelodysplastic syndromes (MDS) treated in the AZA-001 trial.** *Heamtologica* 2010; 95 (s2); 124

12. V. Santini, L. Silverman, J. Seymour, P. Fenaux, G. Mufti, E. Hellström-Lindberg, G. Sanz, C. Finelli, J. Backstrom, D. McKenzie, **C. Beach. Rates of infection and bleeding are not increased in patients (pts) with MDS treated with azacitidine (AZA) compared with best supportive care (BSC).** *Leukemia Research*, Volume 33, Supplement 1, May 2009. Page S133

13. L. Silverman, P. Fenaux, V. Santini, J. Seymour, G. Mufti, E. Hellström-Lindberg, A. Giagounidis, G. Sanz, J. Bennett, J. Backstrom, D. McKenzie, **C. Beach. Transfusion independence (TI) in patients with myelodysplastic syndromes (MDS) treated with azacitidine (AZA).** *Leukemia Research*, Volume 33 Supplement 1, May 2009, Pages S133-S134

14. Herman JG, Gore SD, Mufti GJ, Fenaux P, Santini V, Silverman LR, Seymour JF, Griffiths E, Caraway H, MacBeth KJ, McKenzie D, Backstrom J, **Beach CL. Relationship among gene methylation, azacitidine treatment , and survival in patients with higher-risk myelodysplastic syndromes (MDS): Results from the AZA -001 trial.** Proceedings of the 100th Annual Meeting of the American Association for Cancer Research 2009; April 18-22; Denver, CO: Abstract #4746.

15. Ghulam J Mufti, Steven D. Gore, Valeria Santini, Pierre Fenaux, Lewis R. Silverman, Anne Hagemeijer, Barry Skikne, Eva Hellström-Lindberg, John F. Seymour, **CL Beach**, Jay Backstrom, and Indrasiri Fernando **Influence of Karyotype On Overall Survival in Patients with Higher-Risk Myelodysplastic Syndrome Treated with Azacitidine or a Conventional Care Regimen.** *Blood* (ASH 2009 Annual Meeting Abstracts) 114: 1755.

16. Jeffrey D. Miller, Pierre Fenaux, **C. L. Beach**, Risha Gidwani, and Zeba M. Khan. **Comparative Cost-Effectiveness of Azacitidine Versus Decitabine for the Treatment of Myelodysplastic Syndromes (MDS).** *Blood* (ASH 2009 Annual Meeting Abstracts) 114: 1377.

17. PF Fenaux, L.R. Silverman, G.J Mufti, E. Hellström-Lindberg, V. Santini, N. Gattermann, G. Sanz, J.F. Seymour, S.D. Gore, H. Dombret, D. McKenzie, J. Backstrom, **C.L. Beach. Azacitidine (AZA) Improves overall survival (OS) in who acute myeloid leukemia (AML) in elderly patients with low bone marrow blast counts.** *Hematologica* 2009: 94 (s2); 430

18. J. Seymour, V. Santini, P. Fenaux, A. Giagounidis, G. Sanz, A.F. List, D. McKenzie, J. Backstrom, **C.L. Beach. Effect of Azacitidine (AZA) on transfusion independence (TI) and overall survival (OS) in patients (PTS) with higher-risk myelodysplastic syndromes (MDS).** *Hematologica* 2009; 94 (s2); 328

19. Lewis R. Silverman, Pierre Fenaux, Ghulam J Mufti, Valeria Santini, Eva Hellström-Lindberg, Norbert Gattermann, Guillermo Sanz, Alan F. List, Steven D. Gore, John F Seymour, Jay Backstrom, David McKenzie, and **C. L. Beach. The Effects of Continued Azacitidine (AZA) Treatment Cycles on Response in Higher-Risk Patients (Pts) with Myelodysplastic Syndromes (MDS).** *Blood* (ASH 2008 Annual Meeting Abstracts) 112: 227.

20. Valeria Santini, Pierre Fenaux, Ghulam J Mufti, Eva Hellström-Lindberg, Lewis B. Silverman, Alan List, Steven D. Gore, John F Seymour, Jay Backstrom, David McKenzie, and **C. L Beach. Management and Supportive Care Measures of Adverse Events (AEs) in Higher-Risk MDS Patients (Pts) Treated with Azacitidine (AZA).** *Blood* (ASH 2008 Annual Meeting Abstracts) 112: 1653.

21. John F Seymour, Pierre Fenaux, Lewis B. Silverman, Ghulam J Mufti, Eva Hellström-Lindberg, Valeria Santini, Alan F. List, Steven D. Gore, Jay Backstrom, David McKenzie, and **C. L. Beach. Effects of Azacitidine (AZA) Vs Conventional Care Regimens (CCR) in Elderly (≥75 years) Patients (Pts) with Myelodysplastic Syndromes (MDS) from the AZA-001 Survival Trial .** *Blood* (ASH 2008 Annual Meeting Abstracts) 112: 3629.

22. Pierre Fenaux, Ghulam J Mufti, Eva Hellström-Lindberg, Valeria Santini, Norbert Gattermann, Guillermo Sanz, Alan F. List, Steven D. Gore, John F Seymour, Jay Backstrom, Linda Zimmerman, David McKenzie, **C. L. Beach**, and Lewis B. Silverman **Azacitidine Prolongs Overall Survival (OS) and Reduces Infections and Hospitalizations in Patients (Pts) with WHO-Defined Acute Myeloid Leukemia (AML) Compared with Conventional Care Regimens (CCR)**
Blood (ASH 2008 Annual Meeting Abstracts) 112: 3636.

23. Mufti G, Garcia-Manero G, Hovarth N, Lim Z, Quesnel B, Leone G, Bennett J, Sanz G, McKenzie D, Backstrom J, **Beach CL. Prolonged survival in higher-risk myelodysplastic syndrome (MDS) patients (Pts) with -7/del(7q) treated with azacitidine (AZA).** The European Hematology Association 13th Congress 2008.

24. Fenaux P, Gattermann N, Seymour J, Hellstrom-Lindberg E, Mufti G, Duehrsen U, Gore S, Ramos F, Beyne-Rauzy O, Dombret H, List A, McKenzie D, Backstrom J, Allen A, **Beach CL. Effect of azacitidine (AZA) versus low-dose ara-c (LDAC) on overall survival (OS), hematologic response, transfusion independence, and safety in patients (Pts) with higher-risk myelodysplastic syndromes (MDS).** The European Hematology Association 13th Congress 2008.

25. Santini V,Fenaux P, Vey N, Hofmann, W, Robak T, Bacigalupo AS, Silverman L, Canales M, Scmitz N, Muus P, Fernando I, Backstrom J, Zimmerman L, **Beach CL. European inter-country treatment selection differences do not alter overall survival benefit shown with azacitidine vs conventional care regimens in higher-risk myelodysplastic syndromes.** The European Hematology Association 13th Congress 2008.

26. Lyons R, Cosgriff T, Modi S, McIntyre H, Fernando I, Backstrom J, **Beach CL. Rapid onset of effectiveness with three alternative azacitidine (AZA) dosing regimens in patients (Pts) with myelodysplastic syndromes (MDS).** The European Hematology Association 13th Congress 2008.

27. Santini V, Fenaux P, Mufti GJ, Hellstrom-Lindberg E, List A, Silverman LR, Seymour JF, Backstrom J, McKenzie D, **Beach CL. Patient outcome measures during prolonged survival in patients (Pts) with high-risk myelodysplastic syndromes (MDS) treated with azacitidine (AZA).** American Society of Clinical Oncology 44th Annual Meeting 2008.

28. Mufti GJ, Fenaux P, Hellstrom-Lindberg E, Santini V, List AF, Gore SD, Seymour JF, Silverman LR, McKenzie DR, **Beach CL. Treatment of high-risk MDS patients (pts) with -7/del(7q) with azacitidine (AZA) versus conventional care regimens (CCR): Effects on overall survival (OS).** American Society of Clinical Oncology 44th Annual Meeting 2008.

29. List A, Fenaux P, Mufti GJ, Hellstrom-Lindberg E, Gore SD, Bennett JM, Silverman LR, Backstrom J, Allen A, **Beach CL. Azacitidine (AZA) extends overall survival in higher-risk myelodysplastic syndromes (MDS) without necessity for complete remission**. American Society of Clinical Oncology 44th Annual Meeting 2008.

30. Hellstrom-Lindberg E, Fenaux P, Mufti G, List A, Santini V, Gore S, Seymour J, Backstrom J, McKenzie D, **Beach C. Progression to acute myeloid leukemia (AML) from myelodysplastic syndrome (MDS) is not associated with worsened cytogenetic status.** American Society of Clinical Oncology 44th Annual Meeting 2008.

31. Fenaux P, Mufti GJ, Santini V, Finelli C, Giagounidis A, Schoch R, List AF, Gore SD, Seymour JF, Hellstrom-Lindberg E, Bennett JM, Byrd JC, Backstrom JT, Zimmerman LS, McKenzie DR, **Beach CL, Silverman LR. Azacitidine (AZA) treatment prolongs overall survival (OS) in higher-risk MDS patients compared with conventional care regimens (CCR): Results of the AZA-001 phase III study.** Proceedings from ASH Annual Meeting and Exposition; 2007 Dec 8-11; Atlanta, GA. Abstract 817. *Blood* (ASH Annual Meeting Abstracts) 110: 817.

32. Lyons RM, Cosgriff T, Modi S, McIntyre H, Fernando I, Backstrom J, **Beach CL. Results of the initial treatment phase of a study of three alternative dosing schedules of azacitidine (Vidaza®) in patients with myelodysplastic syndromes (MDS).** Proceedings from ASH Annual Meeting and Exposition; 2007 Dec 8-11; Atlanta, GA. Abstract 819. *Blood* (ASH Annual Meeting Abstracts) 110: 819

33. Lyons RM, Cosgriff T, Modi S, McIntyre H, **Beach CL**, Backstrom JT. **Tolerability and hematologic improvement assessed using three alternative dosing schedules of azacitidine in patients with myelodysplastic syndromes.** *Journal of Clinical Oncology*. 2007 ASCO Annual Meeting Proceedings Part I. Vol 25, No. 18S (June 20 Supplement), 2007. Abstract 7083.

34. Lyons RM, Cosgriff T, Modi S, McIntyre H, **Beach CL,** Backstrom J T. **Hematologic improvement, transfusion independence, and safety assessed using three alternative dosing schedules of azacitidine in patients with myelodysplastic syndromes.** *Blood*. 2006;108(11 Pt 1 of 2):752a. Abstract 2662.

35. Silverman L, McKenzie D, Peterson B, De Castro C, Ellerton J, Knapp K, **Beach C,** Larson R. **Response rates using international working group (IWG) criteria in patients with myelodysplastic syndromes (MDS) treated with azacitidine.** *Blood*. 2005;106(11):709a. Abstract 2526.

36. Lyons R, Cosgriff T, Modi S, Lintz L, **Beach C,** Backstrom J. **Azacitidine (Vidaza®) treatment response assessed using three alternative dosing schedules in patients with myelodysplastic syndromes (MDS).** Session type: poster session 721-II. *Blood*. 2005; 106(11):707a. Abstract 2517.

37. List AF, Byrd JC, Fenaux P, Gore SD, Hellstrom-Lindberg E, Mufti GJ, Seymour JF, Silverman LR, Zimmerman LS, **Beach CL. Comparative trial of azacitidine versus conventional care in high risk MDS patients: methodologic challenges in designing an international survival trial.** *Blood*; 2003:333b-4b.

38. **Beach CL,** Young PM. **Comparison of diltiazem CD versus amlodipine in older female hypertensive patients.** *Clinical Pharmacology and Therapeutics* 1995, 57 (2): 203.

39. Blackshear JL, Stambler BS, Strauss WE, Dias VC, **Beach CL,** Ebner MK. **Control of Heart Rate During transition from IV to oral diltiazem in patients with rapid atrial fibrillation or flutter.** Circulation 1994, 90 (Supp 4):I 375.

40. Young PM, **Beach CL**, Ruckh S, Thadani U. Dose-Response **Evaluation of once daily diltiazem in angina pectoris.** *Clinical Pharmacology and Therapeutics* 1993, 53 (2):139.

41. **Beach CL,** Young PM, Thadani U, Diltiazem Study Group. **Diltiazem plasma concentrations and hemodynamic relationships in stable angina.** *Clinical Pharmacology and Therapeutics* 1991, 49:186.

42. Klein MD, Weiner DA, Cotta LA**, Beach CL. Comparative pharmacodynamics of immediate and sustained release diltiazem.** Circulation 1990, 82 (Supp 3): III 391.

43. Thadani U, Awan N, Kubac G, Silber S, Bittar N, **Beach CL. A multi-center dose response study of sustained release diltiazem hydrochloride in angina pectoris.** *J Am Coll Cardiology* 1990, 15 (Supp A): 172A.

44. Thadani U, Friedman R, Giles T, Weir K, Grossman W, **Beach CL**. **Diltiazem sustained release plus nitrate versus nitrate therapy alone for the treatment of stable angina pectoris.** *J Am Coll Cardiology* 1991, (Supp A): 108A.

45. **Beach CL,** Moore RA, Hewett JE. **Analysis of a multiple-model pharmacokinetic microcomputer program for tobramycin.** *Drug Intelligence and Clinical Pharmacy* 1985; 19:451.

46. Farringer JA, **Beach CL**, Robinson CA, et al. **Quinidine and 3-hydroxyquinidine levels during initiation of anticonvulsant therapy and after chronic anticonvulsant administration.** *Drug Intelligence and Clinical Pharmacy* 1985; 19:461.

47. **Beach CL,** Clementi WA, Campbell AB. **Measurement of lidocaine-free concentrations and free fraction: methodologic consideration.** *Drug Intelligence and Clinical Pharmacy* 1984; 18:506.

48. **Beach CL,** Lenert L, Peck C, et al. **Evaluation of a two-compartment bayesian forecasting program for lidocaine during short and long infusion.** *Clinical Pharmacology and Therapeutics* 1984; 35:228.

49. **Beach CL,** Farringer JA, Bufton MG, et al. **Clinical assessment of a two-compartment bayesian forecasting method for lidocaine in hospitalized patients.** *Drug Intelligence and Clinical Pharmacy* 1983; 17:450.

50. Farringer JA, **Beach CL,** Clementi WA, et al. **Electrocardiographic effects and time course of the phenytoin and quinidine interaction.** *Drug Intelligence and Clinical Pharmacy* 1983; 27:450.

EXHIBIT B

HIGHLIGHTS OF PRESCRIBING INFORMATION

These highlights do not include all the information needed to use VIDAZA safely and effectively. See full prescribing information for VIDAZA.

VIDAZA (azacitidine for injection) for SC or IV use
Initial U.S. Approval: 2004

INDICATIONS AND USAGE

VIDAZA is a nucleoside metabolic inhibitor indicated for the treatment of patients with the following FAB myelodysplastic syndrome (MDS) subtypes: Refractory anemia (RA) or refractory anemia with ringed sideroblasts (RARS) (if accompanied by neutropenia or thrombocytopenia or requiring transfusions), refractory anemia with excess blasts (RAEB), refractory anemia with excess blasts in transformation (RAEB-T), and chronic myelomonocytic leukemia (CMMoL). (1)

DOSAGE AND ADMINISTRATION

- The recommended starting dose for the first treatment cycle, for all patients regardless of baseline hematology values, is VIDAZA 75 mg/m^2 daily for 7 days to be administered by subcutaneous (SC) injection or intravenous (IV) infusion. Premedicate for nausea and vomiting. (2.1)
- Repeat cycles every 4 weeks (2.2). After 2 cycles, may increase dose to 100 mg/m^2 if no beneficial effect is seen and no toxicity other than nausea and vomiting has occurred (2.2). Patients should be treated for a minimum of 4 to 6 cycles. Complete or partial response may require additional treatment cycles (2.2).
- Continue treatment as long as the patient continues to benefit (2.2).
- Patients should be monitored for hematologic response and renal toxicities, with dosage delay or reduction as appropriate (2.3, 2.4, 2.5).

DOSAGE FORMS AND STRENGTHS

- Lyophilized powder in 100 mg single-use vials (3).

CONTRAINDICATIONS

- Advanced malignant hepatic tumors (4.1).
- Hypersensitivity to azacitidine or mannitol (4.2).

WARNINGS AND PRECAUTIONS

- Anemia, neutropenia and thrombocytopenia. Perform complete blood counts (CBC) prior to each treatment cycle and as needed to monitor response and toxicity. (5.1).
- Hepatotoxicity: Use with caution in patients with severe preexisting liver impairment (5.2).
- Renal abnormalities. Monitor patients with renal impairment for toxicity since azacitidine and its metabolites are primarily excreted by the kidneys (5.3).
- Monitor liver chemistries and serum creatinine prior to initiation of therapy and with each cycle (5.4).
- VIDAZA may cause fetal harm when administered to a pregnant woman. Women of childbearing potential should be apprised of the potential hazard to a fetus. (5.5, 8.1).
- Men should be advised not to father a child while receiving VIDAZA (5.6, 13.1).

ADVERSE REACTIONS

Most common adverse reactions (>30%) by SC route are: nausea, anemia, thrombocytopenia, vomiting, pyrexia, leukopenia, diarrhea, injection site erythema, constipation, neutropenia and ecchymosis. Most common adverse reactions by IV route also included petechiae, rigors, weakness and hypokalemia (6.1).

To report SUSPECTED ADVERSE REACTIONS, contact Celgene Corporation at 1-888-423-5436 or FDA at 1-800-FDA-1088 or *www.fda.gov/medwatch*.

DRUG INTERACTIONS

- No formal clinical assessments of drug-drug interactions between VIDAZA and other agents have been conducted (7).

USE IN SPECIFIC POPULATIONS

- Nursing Mothers: Discontinue drug or nursing taking into consideration importance of drug to mother (8.3).
- Because elderly patients are more likely to have decreased renal function, it may be useful to monitor renal function (8.5).

See 17 for PATIENT COUNSELING INFORMATION.

Revised: December 2012

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FULL PRESCRIBING INFORMATION

1 INDICATIONS AND USAGE

1.1 Myelodysplastic Syndromes (MDS)

VIDAZA® is indicated for treatment of patients with the following French-American-British (FAB) myelodysplastic syndrome subtypes: refractory anemia (RA) or refractory anemia with ringed sideroblasts (if accompanied by neutropenia or thrombocytopenia or requiring transfusions), refractory anemia with excess blasts (RAEB), refractory anemia with excess blasts in transformation (RAEB-T), and chronic myelomonocytic leukemia (CMMoL).

2 DOSAGE AND ADMINISTRATION

2.1 First Treatment Cycle

The recommended starting dose for the first treatment cycle, for all patients regardless of baseline hematology laboratory values, is 75 mg/m^2 subcutaneously or intravenously, daily for 7 days. Patients should be premedicated for nausea and vomiting.

2.2 Subsequent Treatment Cycles

Cycles should be repeated every 4 weeks. The dose may be increased to 100 mg/m^2 if no beneficial effect is seen after 2 treatment cycles and if no toxicity other than nausea and vomiting has occurred. It is recommended that patients be treated for a minimum of 4 to 6 cycles. However, complete or partial response may require additional treatment cycles. Treatment may be continued as long as the patient continues to benefit.

Patients should be monitored for hematologic response and renal toxicities *[see Warnings and Precautions (5.3)]*, and dosage delay or reduction as described below may be necessary.

2.3 Dosage Adjustment Based on Hematology Laboratory Values

- For patients with baseline (start of treatment) WBC ≥3.0 x10^9/L, ANC ≥1.5 x10^9/L, and platelets ≥75.0 x10^9/L, adjust the dose as follows, based on nadir counts for any given cycle:

Nadir Counts		% Dose in the Next Course
ANC (x10^9/L)	Platelets (x10^9/L)	
<0.5	<25.0	50%
0.5–1.5	25.0-50.0	67%
>1.5	>50.0	100%

- For patients whose baseline counts are WBC <3.0 x10^9/L, ANC<1.5 x10^9/L, or platelets <75.0 x10^9/L, dose adjustments should be based on nadir counts and bone marrow biopsy cellularity at the time of the nadir as noted below, unless there is clear improvement in differentiation (percentage of mature granulocytes is higher and ANC is higher than at onset of that course) at the time of the next cycle, in which case the dose of the current treatment should be continued.

WBC or Platelet Nadir % decrease in counts from baseline	Bone Marrow Biopsy Cellularity at Time of Nadir (%)		
	30-60	15-30	<15
	% Dose in the Next Course		
50 - 75	100	50	33
>75	75	50	33

If a nadir as defined in the table above has occurred, the next course of treatment should be given 28 days after the start of the preceding course, provided that both the WBC and the platelet counts are >25% above the nadir and rising. If a >25% increase above the nadir is not seen by day 28, counts should be reassessed every 7 days. If a 25% increase is not seen by day 42, then the patient should be treated with 50% of the scheduled dose.

2.4 Dosage Adjustment Based on Renal Function and Serum Electrolytes

If unexplained reductions in serum bicarbonate levels to <20 mEq/L occur, the dosage should be reduced by 50% on the next course. Similarly, if unexplained elevations of BUN or serum creatinine occur, the next cycle should be delayed until values return to normal or baseline and the dose should be reduced by 50% on the next treatment course *[see Warnings and Precautions (5.3)]*.

2.5 Use in Geriatric Patients

Azacitidine and its metabolites are known to be substantially excreted by the kidney, and the risk of toxic reactions to this drug may be greater in patients with impaired renal function. Because elderly patients are more likely to have decreased renal function, care should be taken in dose selection, and it may be useful to monitor renal function *[see Warnings and Precautions (5.3) and Use in Specific Populations (8.5)]*.

2.6 Preparation of VIDAZA

VIDAZA is a cytotoxic drug and, as with other potentially toxic compounds, caution should be exercised when handling and preparing VIDAZA suspensions *[see How Supplied/Storage and Handling (16)]*.

If reconstituted VIDAZA comes into contact with the skin, immediately and thoroughly wash with soap and water. If it comes into contact with mucous membranes, flush thoroughly with water.

The VIDAZA vial is single-use and does not contain any preservatives. Unused portions of each vial should be discarded properly *[see How Supplied/Storage and Handling (16)]*. Do not save any unused portions for later administration.

2.7 Instructions for Subcutaneous Administration

VIDAZA should be reconstituted aseptically with 4 mL sterile water for injection. The diluent should be injected slowly into the vial. Vigorously shake or roll the vial until a uniform suspension is achieved. The suspension will be cloudy. The resulting suspension will contain azacitidine 25 mg/mL. Do not filter the suspension after reconstitution. Doing so could remove the active substance.

Preparation for Immediate Subcutaneous Administration: Doses greater than 4 mL should be divided equally into 2 syringes. The product may be held at room temperature for up to 1 hour, but must be administered within 1 hour after reconstitution.

Preparation for Delayed Subcutaneous Administration: The reconstituted product may be kept in the vial or drawn into a syringe. Doses greater than 4 mL should be divided equally into 2 syringes. The product must be refrigerated immediately. When VIDAZA is reconstituted using water for injection that has not been refrigerated, the reconstituted product may be held under refrigerated conditions (2°C - 8°C, 36°F - 46°F) for up to 8 hours. When VIDAZA is reconstituted using refrigerated (2°C - 8°C, 36°F - 46°F) water for injection, the reconstituted product may be stored under refrigerated conditions (2°C - 8°C, 36°F - 46°F) for up to 22 hours. After removal from refrigerated conditions, the suspension may be allowed to equilibrate to room temperature for up to 30 minutes prior to administration.

Subcutaneous Administration

To provide a homogeneous suspension, the contents of the dosing syringe must be re-suspended immediately prior to administration. To re-suspend, vigorously roll the syringe between the palms until a uniform, cloudy suspension is achieved.

VIDAZA suspension is administered subcutaneously. Doses greater than 4 mL should be divided equally into 2 syringes and injected into 2 separate sites. Rotate sites for each injection (thigh, abdomen, or upper arm). New injections should be given at least one inch from an old site and never into areas where the site is tender, bruised, red, or hard.

Suspension Stability: VIDAZA reconstituted with non-refrigerated water for injection for subcutaneous administration may be stored for up to 1 hour at 25°C (77°F) or for up to 8 hours between 2°C and 8°C (36°F and 46°F); when reconstituted with refrigerated (2°C - 8°C, 36°F - 46°F) water for injection, it may be stored for 22 hours between 2°C and 8°C (36°F and 46°F).

2.8 Instructions for Intravenous Administration

Reconstitute the appropriate number of VIDAZA vials to achieve the desired dose. Reconstitute each vial with 10 mL sterile water for injection. Vigorously shake or roll the vial until all solids are dissolved. The resulting solution will contain azacitidine 10 mg/mL. The solution should be clear. Parenteral drug product should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

Withdraw the required amount of VIDAZA solution to deliver the desired dose and inject into a 50 -100 mL infusion bag of either 0.9% Sodium Chloride Injection or Lactated Ringer's Injection.

Intravenous Solution Incompatibility

VIDAZA is incompatible with 5% Dextrose solutions, Hespan, or solutions that contain bicarbonate. These solutions have the potential to increase the rate of degradation of VIDAZA and should therefore be avoided.

Intravenous Administration

VIDAZA solution is administered intravenously. Administer the total dose over a period of 10 - 40 minutes. The administration must be completed within 1 hour of reconstitution of the VIDAZA vial.

Solution Stability: VIDAZA reconstituted for intravenous administration may be stored at 25°C (77°F), but administration must be completed within 1 hour of reconstitution.

3 DOSAGE FORMS AND STRENGTHS

VIDAZA (azacitidine for injection) is supplied as lyophilized powder in 100 mg single-use vials.

4 CONTRAINDICATIONS

4.1 Advanced Malignant Hepatic Tumors

VIDAZA is contraindicated in patients with advanced malignant hepatic tumors *[see Warnings and Precautions (5.2)]*.

4.2 Hypersensitivity to Azacitidine or Mannitol

VIDAZA is contraindicated in patients with a known hypersensitivity to azacitidine or mannitol.

5 WARNINGS AND PRECAUTIONS

5.1 Anemia, Neutropenia and Thrombocytopenia

Treatment with VIDAZA is associated with anemia, neutropenia and thrombocytopenia. Complete blood counts should be performed as needed to monitor response and toxicity, but at a minimum, prior to each dosing cycle. After administration of the recommended dosage for the first cycle, dosage for subsequent cycles should be reduced or delayed based on nadir counts and hematologic response *[see Dosage and Administration (2.3)]*.

5.2 Severe Pre-existing Hepatic Impairment

Because azacitidine is potentially hepatotoxic in patients with severe pre-existing hepatic impairment, caution is needed in patients with liver disease. Patients with extensive tumor burden due to metastatic disease have been reported to experience progressive hepatic coma and death during azacitidine treatment, especially in such patients with baseline albumin <30 g/L. Azacitidine is contraindicated in patients with advanced malignant hepatic tumors *[see Contraindications (4.1)]*.

Safety and effectiveness of VIDAZA in patients with MDS and hepatic impairment have not been studied as these patients were excluded from the clinical trials.

5.3 Renal Abnormalities

Renal abnormalities ranging from elevated serum creatinine to renal failure and death have been reported in patients treated with intravenous azacitidine in combination with other chemotherapeutic agents for nonMDS conditions. In addition, renal tubular acidosis, defined as a fall in serum bicarbonate to <20 mEq/L in association with an alkaline urine and hypokalemia (serum potassium <3 mEq/L) developed in 5 patients with CML treated with azacitidine and etoposide. If unexplained reductions in serum bicarbonate <20 mEq/L or elevations of BUN or serum creatinine occur, the dosage should be reduced or held *[see Dosage and Administration (2.4)]*.

Patients with renal impairment should be closely monitored for toxicity since azacitidine and its metabolites are primarily excreted by the kidneys *[see Dosage and Administration (2.4, 2.5)]*.

Safety and effectiveness of VIDAZA in patients with MDS and renal impairment have not been studied as these patients were excluded from the clinical trials.

5.4 Monitoring Laboratory Tests

Complete blood counts should be performed as needed to monitor response and toxicity, but at a minimum, prior to each cycle. Liver chemistries and serum creatinine should be obtained prior to initiation of therapy.

5.5 Use in Pregnancy

VIDAZA may cause fetal harm when administered to a pregnant woman. Azacitidine caused congenital malformations in animals. Women of childbearing potential should be advised to avoid pregnancy during treatment with VIDAZA. There are no adequate and well-controlled studies in pregnant women using VIDAZA. If this drug is used during pregnancy or if a patient becomes pregnant while taking this drug, the patient should be apprised of the potential hazard to the fetus *[see Use in Specific Populations (8.1)]*.

5.6 Use in Males

Men should be advised to not father a child while receiving treatment with VIDAZA. In animal studies, pre-conception treatment of male mice and rats resulted in increased embryofetal loss in mated females *[see Nonclinical Toxicology (13)]*.

6 ADVERSE REACTIONS

6.1 Overview

Adverse Reactions Described in Other Labeling Sections: anemia, neutropenia, thrombocytopenia, elevated serum creatinine, renal failure, renal tubular acidosis, hypokalemia, hepatic coma *[see Warnings and Precautions (5.1, 5.2, 5.3)]*.

Most Commonly Occurring Adverse Reactions (SC or IV Route): nausea, anemia, thrombocytopenia, vomiting, pyrexia, leukopenia, diarrhea, injection site erythema, constipation, neutropenia, ecchymosis. The most common adverse reactions by IV route also included petechiae, rigors, weakness and hypokalemia.

Adverse Reactions Most Frequently (>2%) Resulting in Clinical Intervention (SC or IV Route):
Discontinuation: leukopenia, thrombocytopenia, neutropenia.
Dose Held: leukopenia, neutropenia, thrombocytopenia, pyrexia, pneumonia, febrile neutropenia.
Dose Reduced: leukopenia, neutropenia, thrombocytopenia.

6.2 Adverse Reactions in Clinical Trials

Because clinical trials are conducted under widely varying conditions, adverse reaction rates observed in the clinical trials of a drug cannot be directly compared to rates in the clinical trials of another drug and may not reflect the rates observed in practice.

The data described below reflect exposure to VIDAZA in 443 MDS patients from 4 clinical studies. Study 1 was a supportive-care controlled trial (SC administration), Studies 2 and 3 were single arm studies (one with SC administration and one with IV administration), and Study 4 was an international randomized trial (SC administration) *[see Clinical Studies (14)]*.

In Studies 1, 2 and 3, a total of 268 patients were exposed to VIDAZA, including 116 exposed for 6 cycles (approximately 6 months) or more and 60 exposed for greater than 12 cycles (approximately one year). VIDAZA was studied primarily in supportive-care controlled and uncontrolled trials (n=150 and n=118, respectively). The population in the subcutaneous studies (n=220) was 23 to 92 years old (mean 66.4 years), 68% male, and 94% white, and had MDS or AML. The population in the IV study (n=48) was 35 to 81 years old (mean 63.1 years), 65% male, and 100% white. Most patients received average daily doses between 50 and 100 mg/m^2.

In Study 4, a total of 175 patients with higher-risk MDS (primarily RAEB and RAEB-T subtypes) were exposed to VIDAZA. Of these patients, 119 were exposed for 6 or more cycles, and 63 for at least 12 cycles. The mean age of this population was 68.1 years (ranging from 42 to 83 years), 74% were male, and 99% were white. Most patients received daily VIDAZA doses of 75 mg/m^2.

Table 1 presents adverse reactions occurring in at least 5% of patients treated with VIDAZA (SC) in Studies 1 and 2. It is important to note that duration of exposure was longer for the VIDAZA-treated group than for the observation group: patients received VIDAZA for a mean of 11.4 months while mean time in the observation arm was 6.1 months.

Table 1: Most Frequently Observed Adverse Reactions (≥ 5.0% in All SC VIDAZA Treated Patients; Studies 1 and 2)

	Number (%) of Patients	
System Organ Class Preferred Term[a]	**All VIDAZA[b] (N=220)**	**Observation[c] (N=92)**
Blood and lymphatic system disorders		
Anemia	153 (69.5)	59 (64.1)
Anemia aggravated	12 (5.5)	5 (5.4)
Febrile neutropenia	36 (16.4)	4 (4.3)
Leukopenia	106 (48.2)	27 (29.3)
Neutropenia	71 (32.3)	10 (10.9)
Thrombocytopenia	144 (65.5)	42 (45.7)
Gastrointestinal disorders		
Abdominal tenderness	26 (11.8)	1 (1.1)
Constipation	74 (33.6)	6 (6.5)
Diarrhea	80 (36.4)	13 (14.1)
Gingival bleeding	21 (9.5)	4 (4.3)
Loose stools	12 (5.5)	0
Mouth hemorrhage	11 (5.0)	1 (1.1)
Nausea	155 (70.5)	16 (17.4)
Stomatitis	17 (7.7)	0
Vomiting	119 (54.1)	5 (5.4)
General disorders and administration site conditions		
Chest pain	36 (16.4)	5 (5.4)
Injection site bruising	31 (14.1)	0
Injection site erythema	77 (35.0)	0
Injection site granuloma	11 (5.0)	0
Injection site pain	50 (22.7)	0
Injection site pigmentation changes	11 (5.0)	0
Injection site pruritus	15 (6.8)	0
Injection site reaction	30 (13.6)	0
Injection site swelling	11 (5.0)	0
Lethargy	17 (7.7)	2 (2.2)
Malaise	24 (10.9)	1 (1.1)
Pyrexia	114 (51.8)	28 (30.4)
Infections and infestations		
Nasopharyngitis	32 (14.5)	3 (3.3)
Pneumonia	24 (10.9)	5 (5.4)
Upper respiratory tract infection	28 (12.7)	4 (4.3)
Injury, poisoning, and procedural complications		
Post procedural hemorrhage	13 (5.9)	1 (1.1)
Metabolism and nutrition disorders		
Anorexia	45 (20.5)	6 (6.5)
Musculoskeletal and connective tissue disorders		
Arthralgia	49 (22.3)	3 (3.3)
Chest wall pain	11 (5.0)	0
Myalgia	35 (15.9)	2 (2.2)
Nervous system disorders		
Dizziness	41 (18.6)	5 (5.4)
Headache	48 (21.8)	10 (10.9)
Psychiatric disorders		
Anxiety	29 (13.2)	3 (3.3)
Insomnia	24 (10.9)	4 (4.3)

continued

Table 1: Most Frequently Observed Adverse Reactions (≥ 5.0% in All SC VIDAZA Treated Patients; Studies 1 and 2)

	Number (%) of Patients	
System Organ Class Preferred Term[a]	**All VIDAZA[b] (N=220)**	**Observation[c] (N=92)**
Respiratory, thoracic and mediastinal disorders		
Dyspnea	64 (29.1)	11 (12.0)
Skin and subcutaneous tissue disorders		
Dry skin	11 (5.0)	1 (1.1)
Ecchymosis	67 (30.5)	14 (15.2)
Erythema	37 (16.8)	4 (4.3)
Rash	31 (14.1)	9 (9.8)
Skin nodule	11 (5.0)	1 (1.1)
Urticaria	13 (5.9)	1 (1.1)
Vascular disorders		
Hematoma	19 (8.6)	0
Hypotension	15 (6.8)	2 (2.2)
Petechiae	52 (23.6)	8 (8.7)

[a] Multiple terms of the same preferred terms for a patient are only counted once within each treatment group.
[b] Includes adverse reactions from all patients exposed to VIDAZA, including patients after crossing over from observations.
[c] Includes adverse reactions from observation period only; excludes any adverse events after crossover to VIDAZA.

Table 2 presents adverse reactions occurring in at least 5% of patients treated with VIDAZA in Study 4. Similar to Studies 1 and 2 described above, duration of exposure to treatment with VIDAZA was longer (mean 12.2 months) compared with best supportive care (mean 7.5 months).

Table 2: Most Frequently Observed Adverse Reactions (≥ 5.0% in the VIDAZA Treated Patients and the Percentage with NCI CTC Grade 3/4 Reactions; Study 4)

	Number (%) of Patients			
	Any Grade		Grade 3/4	
System Organ Class Preferred Term[a]	**VIDAZA (N=175)**	**Best Supportive Care Only (N=102)**	**VIDAZA (N=175)**	**Best Supportive Care Only (N=102)**
Blood and lymphatic system disorders				
Anemia	90 (51.4)	45 (44.1)	24 (13.7)	9 (8.8)
Febrile neutropenia	24 (13.7)	10 (9.8)	22 (12.6)	7 (6.9)
Leukopenia	32 (18.3)	2 (2.0)	26 (14.9)	1 (1.0)
Neutropenia	115 (65.7)	29 (28.4)	107 (61.1)	22 (21.6)
Thrombocytopenia	122 (69.7)	35 (34.3)	102 (58.3)	29 (28.4)
Gastrointestinal disorders				
Abdominal pain	22 (12.6)	7 (6.9)	7 (4.0)	0
Constipation	88 (50.3)	8 (7.8)	2 (1.1)	0
Dyspepsia	10 (5.7)	2 (2.0)	0	0
Nausea	84 (48.0)	12 (11.8)	3 (1.7)	0
Vomiting	47 (26.9)	7 (6.9)	0	0
General disorders and administration site conditions				
Fatigue	42 (24.0)	12 (11.8)	6 (3.4)	2 (2.0)
Injection site bruising	9 (5.1)	0	0	0
Injection site erythema	75 (42.9)	0	0	0
Injection site hematoma	11 (6.3)	0	0	0
Injection site induration	9 (5.1)	0	0	0
Injection site pain	33 (18.9)	0	0	0
Injection site rash	10 (5.7)	0	0	0
Injection site reaction	51 (29.1)	0	1 (0.6)	0
Pyrexia	53 (30.3)	18 (17.6)	8 (4.6)	1 (1.0)
Infections and infestations				
Rhinitis	10 (5.7)	1 (1.0)	0	0
Upper respiratory tract infection	16 (9.1)	4 (3.9)	3 (1.7)	0
Urinary tract infection	15 (8.6)	3 (2.9)	3 (1.7)	0

continued

Table 2: Most Frequently Observed Adverse Reactions (≥ 5.0% in the VIDAZA Treated Patients and the Percentage with NCI CTC Grade 3/4 Reactions; Study 4)

	Number (%) of Patients			
	Any Grade		Grade 3/4	
System Organ Class Preferred Term[a]	**VIDAZA (N=175)**	**Best Supportive Care Only (N=102)**	**VIDAZA (N=175)**	**Best Supportive Care Only (N=102)**
Investigations				
Weight decreased	14 (8.0)	0	1 (0.6)	0
Metabolism and nutrition disorders				
Hypokalemia	11 (6.3)	3 (2.9)	3 (1.7)	3 (2.9)
Nervous system disorders				
Lethargy	13 (7.4)	2 (2.0)	0	1 (1.0)
Psychiatric disorders				
Anxiety	9 (5.1)	1 (1.0)	0	0
Insomnia	15 (8.6)	3 (2.9)	0	0
Renal and urinary disorders				
Hematuria	11 (6.3)	2 (2.0)	4 (2.3)	1 (1.0)
Respiratory, thoracic and mediastinal disorders				
Dyspnea	26 (14.9)	5 (4.9)	6 (3.4)	2 (2.0)
Dyspnea exertional	9 (5.1)	1 (1.0)	0	0
Pharyngolaryngeal pain	11 (6.3)	3 (2.9)	0	0
Skin and subcutaneous tissue disorders				
Erythema	13 (7.4)	3 (2.9)	0	0
Petechiae	20 (11.4)	4 (3.9)	2 (1.1)	0
Pruritus	21 (12.0)	2 (2.0)	0	0
Rash	18 (10.3)	1 (1.0)	0	0
Vascular disorders				
Hypertension	15 (8.6)	4 (3.9)	2 (1.1)	2 (2.0)

[a] Multiple reports of the same preferred term from a patient were only counted once within each treatment.

In Studies 1, 2 and 4 with SC administration of VIDAZA, adverse reactions of neutropenia, thrombocytopenia, anemia, nausea, vomiting, diarrhea, constipation, and injection site erythema/reaction tended to increase in incidence with higher doses of VIDAZA. Adverse reactions that tended to be more pronounced during the first 1 to 2 cycles of SC treatment compared with later cycles included thrombocytopenia, neutropenia, anemia, nausea, vomiting, injection site erythema/pain/bruising/reaction, constipation, petechiae, dizziness, anxiety, hypokalemia, and insomnia. There did not appear to be any adverse reactions that increased in frequency over the course of treatment.

Overall, adverse reactions were qualitatively similar between the IV and SC studies. Adverse reactions that appeared to be specifically associated with the IV route of administration included infusion site reactions (e.g. erythema or pain) and catheter site reactions (e.g. infection, erythema, or hemorrhage).

In clinical studies of either SC or IV VIDAZA, the following serious adverse reactions occurring at a rate of < 5% (and not described in Tables 1 or 2) were reported:

Blood and lymphatic system disorders: agranulocytosis, bone marrow failure, pancytopenia splenomegaly.

Cardiac disorders: atrial fibrillation, cardiac failure, cardiac failure congestive, cardio-respiratory arrest, congestive cardiomyopathy.

Eye disorders: eye hemorrhage

Gastrointestinal disorders: diverticulitis, gastrointestinal hemorrhage, melena, perirectal abscess.

General disorders and administration site conditions: catheter site hemorrhage, general physical health deterioration, systemic inflammatory response syndrome.

Hepatobiliary disorders: cholecystitis.

Immune system disorders: anaphylactic shock, hypersensitivity.

Infections and infestations: abscess limb, bacterial infection, cellulitis, blastomycosis, injection site infection, Klebsiella sepsis, neutropenic sepsis, pharyngitis streptococcal, pneumonia Klebsiella, sepsis, septic shock, Staphylococcal bacteremia, Staphylococcal infection, toxoplasmosis.

Metabolism and nutrition disorders: dehydration.

Musculoskeletal and connective tissue disorders: bone pain aggravated, muscle weakness, neck pain.

Neoplasms benign, malignant and unspecified: leukemia cutis.

Nervous system disorders: cerebral hemorrhage, convulsions, intracranial hemorrhage.

Renal and urinary disorders: loin pain, renal failure.

Respiratory, thoracic and mediastinal disorders: hemoptysis, lung infiltration, pneumonitis, respiratory distress.

Skin and subcutaneous tissue disorders: pyoderma gangrenosum, rash pruritic, skin induration.

Surgical and medical procedures: cholecystectomy.

Vascular disorders: orthostatic hypotension.

6.3 Postmarketing Experience

The following adverse reactions have been identified during postmarketing use of VIDAZA. Because these reactions are reported voluntarily from a population of uncertain size, it is not always possible to reliably estimate their frequency or establish a causal relationship to drug exposure.

- Interstitial lung disease
- Tumor lysis syndrome
- Injection site necrosis
- Sweet's syndrome (acute febrile neutrophilic dermatosis)

7 DRUG INTERACTIONS

No formal clinical assessments of drug-drug interactions between VIDAZA and other agents have been conducted *[see Clinical Pharmacology (12.3)]*.

8 USE IN SPECIFIC POPULATIONS

8.1 Pregnancy

Pregnancy Category D *[see Warning and Precautions (5.5)]*

VIDAZA may cause fetal harm when administered to a pregnant woman. Azacitidine was teratogenic in animals. There are no adequate and well controlled studies with VIDAZA in pregnant women. Women of childbearing potential should be advised to avoid pregnancy during treatment with VIDAZA. If this drug is used during pregnancy or if a patient becomes pregnant while taking this drug, the patient should be apprised of the potential hazard to the fetus.

Female partners of male patients receiving VIDAZA should not become pregnant *[see Nonclinical Toxicology (13.1)]*.

Early embryotoxicity studies in mice revealed a 44% frequency of intrauterine embryonal death (increased resorption) after a single IP (intraperitoneal) injection of 6 mg/m^2 (approximately 8% of the recommended human daily dose on a mg/m^2 basis) azacitidine on gestation day 10. Developmental abnormalities in the brain have been detected in mice given azacitidine on or before gestation day 15 at doses of ~3-12 mg/m^2 (approximately 4%-16% the recommended human daily dose on a mg/m^2 basis).

In rats, azacitidine was clearly embryotoxic when given IP on gestation days 4-8 (postimplantation) at a dose of 6 mg/m^2 (approximately 8% of the recommended human daily dose on a mg/m^2 basis), although treatment in the preimplantation period (on gestation days 1-3) had no adverse effect on the embryos. Azacitidine caused multiple fetal abnormalities in rats after a single IP dose of 3 to 12 mg/m^2 (approximately 8% the recommended human daily dose on a mg/m^2 basis) given on gestation day 9, 10, 11 or 12. In this study azacitidine caused fetal death when administered at 3-12 mg/m^2 on gestation days 9 and 10; average live animals per litter was reduced to 9% of control at the highest dose on gestation day 9. Fetal anomalies included: CNS anomalies (exencephaly/encephalocele), limb anomalies (micromelia, club foot, syndactyly, oligodactyly), and others (micrognathia, gastroschisis, edema, and rib abnormalities).

8.3 Nursing Mothers

It is not known whether azacitidine or its metabolites are excreted in human milk. Because many drugs are excreted in human milk and because of the potential for tumorigenicity shown for azacitidine in animal studies and the potential for serious adverse reactions in nursing infants from VIDAZA, a decision should be made whether to discontinue nursing or to discontinue the drug, taking into consideration the importance of the drug to the mother.

8.4 Pediatric Use

Safety and effectiveness in pediatric patients have not been established.

8.5 Geriatric Use

Of the total number of patients in Studies 1, 2 and 3, 62% were 65 years and older and 21% were 75 years and older. No overall differences in effectiveness were observed between these patients and younger patients. In addition there were no relevant differences in the frequency of adverse reactions observed in patients 65 years and older compared to younger patients.

Of the 179 patients randomized to azacitidine in Study 4, 68% were 65 years and older and 21% were 75 years and older. Survival data for patients 65 years and older were consistent with overall survival results. The majority of adverse reactions occurred at similar frequencies in patients < 65 years of age and patients 65 years of age and older.

Azacitidine and its metabolites are known to be substantially excreted by the kidney, and the risk of adverse reactions to this drug may be greater in patients with impaired renal function. Because elderly patients are more likely to have decreased renal function, it may be useful to monitor renal function *[see Dosage and Administration (2.5) and Warnings and Precautions (5.3)]*.

8.6 Gender

There were no clinically relevant differences in safety and efficacy based on gender.

8.7 Race

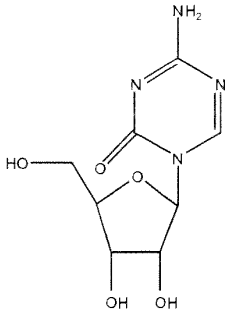
Greater than 90% of all patients in all trials were Caucasian. Therefore, no comparisons between Caucasians and non-Caucasians were possible.

10 OVERDOSAGE

One case of overdose with VIDAZA was reported during clinical trials. A patient experienced diarrhea, nausea, and vomiting after receiving a single IV dose of approximately 290 mg/m^2, almost 4 times the recommended starting dose. The events resolved without sequelae, and the correct dose was resumed the following day. In the event of overdosage, the patient should be monitored with appropriate blood counts and should receive supportive treatment, as necessary. There is no known specific antidote for VIDAZA overdosage.

11 DESCRIPTION

VIDAZA (azacitidine for injection) contains azacitidine, which is a pyrimidine nucleoside analog of cytidine. Azacitidine is 4-amino-1-β-D-ribofuranosyl-s-triazin-2(1H)-one. The structural formula is as follows:



The empirical formula is $C_8H_{12}N_4O_5$. The molecular weight is 244. Azacitidine is a white to off-white solid. Azacitidine was found to be insoluble in acetone, ethanol, and methyl ethyl ketone; slightly soluble in ethanol/water (50/50), propylene glycol, and polyethylene glycol; sparingly soluble in water, water saturated octanol, 5% dextrose in water, N-methyl-2-pyrrolidone, normal saline and 5% Tween 80 in water; and soluble in dimethylsulfoxide (DMSO).

The finished product is supplied in a sterile form for reconstitution as a suspension for subcutaneous injection or reconstitution as a solution with further dilution for intravenous infusion. Vials of VIDAZA contain 100 mg of azacitidine and 100 mg mannitol as a sterile lyophilized powder.

12 CLINICAL PHARMACOLOGY

12.1 Mechanism of Action

VIDAZA is a pyrimidine nucleoside analog of cytidine. VIDAZA is believed to exert its antineoplastic effects by causing hypomethylation of DNA and direct cytotoxicity on abnormal hematopoietic cells in the bone marrow. The concentration of azacitidine required for maximum inhibition of DNA methylation *in vitro* does not cause major suppression of DNA synthesis. Hypomethylation may restore normal function to genes that are critical for differentiation and proliferation. The cytotoxic effects of azacitidine cause the death of rapidly dividing cells, including cancer cells that are no longer responsive to normal growth control mechanisms. Non-proliferating cells are relatively insensitive to azacitidine.

12.3 Pharmacokinetics

The pharmacokinetics of azacitidine were studied in 6 MDS patients following a single 75 mg/m^2 subcutaneous (SC) dose and a single 75 mg/m^2 intravenous (IV) dose. Azacitidine is rapidly absorbed after SC administration; the peak plasma azacitidine concentration of 750 ± 403 ng/ml occurred in 0.5 hour. The bioavailability of SC azacitidine relative to IV azacitidine is approximately 89%, based on area under the curve. Mean volume of distribution following IV dosing is 76 ± 26 L. Mean apparent SC clearance is 167 ± 49 L/hour and mean half-life after SC administration is 41 ± 8 minutes.

Published studies indicate that urinary excretion is the primary route of elimination of azacitidine and its metabolites. Following IV administration of radioactive azacitidine to 5 cancer patients, the cumulative urinary excretion was 85% of the radioactive dose. Fecal excretion accounted for <1% of administered radioactivity over 3 days. Mean excretion of radioactivity in urine following SC administration of ^{14}C-azacitidine was 50%. The mean elimination half-lives of total radioactivity (azacitidine and its metabolites) were similar after IV and SC administrations, about 4 hours.

Special Populations

The effects of renal or hepatic impairment, gender, age, or race on the pharmacokinetics of azacitidine have not been studied *[see Dosage and Administration (2.4), Contraindications (4.1) and Warnings and Precautions (5.2, 5.3)]*.

Drug-Drug Interactions

No formal clinical drug interaction studies with azacitidine have been conducted.

An *in vitro* study of azacitidine incubation in human liver fractions indicated that azacitidine may be metabolized by the liver. Whether azacitidine metabolism may be affected by known microsomal enzyme inhibitors or inducers has not been studied.

An *in vitro* study with cultured human hepatocytes indicated that azacitidine at concentrations up to 100 µM (IV Cmax = 10.6 µM) does not cause any inhibition of CYP2B6 and CYP2C8. The potential of azacitidine to inhibit other cytochrome P450 (CYP) enzymes is not known.

In vitro studies with human cultured hepatocytes indicate that azacitidine at concentrations of 1.0 µM to 100 µM does not induce CYP 1A2, 2C19, or 3A4/5.

13 NONCLINICAL TOXICOLOGY

13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility

The potential carcinogenicity of azacitidine was evaluated in mice and rats. Azacitidine induced tumors of the hematopoietic system in female mice at 2.2 mg/kg (6.6 mg/m^2, approximately 8% the recommended human daily dose on a mg/m^2 basis) administered IP three times per week for 52 weeks. An increased incidence of tumors in the lymphoreticular system, lung, mammary gland, and skin was seen in mice treated with azacitidine IP at 2.0 mg/kg (6.0 mg/m^2, approximately 8% the recommended human daily dose on a mg/m^2 basis) once a week for 50 weeks. A tumorigenicity study in rats dosed twice weekly at 15 or 60 mg/m^2 (approximately 20-80% the recommended human daily dose on a mg/m^2 basis) revealed an increased incidence of testicular tumors compared with controls.

The mutagenic and clastogenic potential of azacitidine was tested in *in vitro* bacterial systems Salmonella typhimurium strains TA100 and several strains of trpE8, Escherichia coli strains WP14 Pro, WP3103P, WP3104P, and CC103; in *in vitro* forward gene mutation assay in mouse lymphoma cells and human lymphoblast cells; and in an *in vitro* micronucleus assay in mouse L5178Y lymphoma cells and Syrian hamster embryo cells. Azacitidine was mutagenic in bacterial and mammalian cell systems. The clastogenic effect of azacitidine was shown by the induction of micronuclei in L5178Y mouse cells and Syrian hamster embryo cells.

Administration of azacitidine to male mice at 9.9 mg/m^2 (approximately 9% the recommended human daily dose on a mg/m^2 basis) daily for 3 days prior to mating with untreated female mice resulted in decreased fertility and loss of offspring during subsequent embryonic and postnatal development. Treatment of male rats 3 times per week for 11 or 16 weeks at doses of 15-30 mg/m^2 (approximately 20-40%, the recommended human daily dose on a mg/m^2 basis) resulted in decreased weight of the testes and epididymides, and decreased sperm counts accompanied by decreased pregnancy rates and increased loss of embryos in mated females. In a related study, male rats treated for 16 weeks at 24 mg/m^2 resulted in an increase in abnormal embryos in mated females when examined on day 2 of gestation.

14 CLINICAL STUDIES

Myelodysplastic Syndromes (MDS)

Study 1 was a randomized, open-label, controlled trial carried out in 53 U.S. sites compared the safety and efficacy of subcutaneous VIDAZA plus supportive care with supportive care alone ("observation") in patients with any of the five FAB subtypes of myelodysplastic syndromes (MDS): refractory anemia (RA), RA with ringed sideroblasts (RARS), RA with excess blasts (RAEB), RAEB in transformation (RAEB-T), and chronic myelomonocytic leukemia (CMMoL). RA and RARS patients were included if they met one or more of the following criteria: required packed RBC transfusions; had platelet counts ≤50.0 x 10^9/L; required platelet transfusions; or were neutropenic (ANC <1.0 x 10^9/L) with infections requiring treatment with antibiotics. Patients with acute myelogenous leukemia (AML) were not intended to be included. Supportive care allowed in this study included blood transfusion products, antibiotics, antiemetics, analgesics and antipyretics. The use of hematopoeitic growth factors was prohibited. Baseline patient and disease characteristics are summarized in Table 3; the 2 groups were similar.

VIDAZA was administered at a subcutaneous dose of 75 mg/m^2 daily for 7 days every 4 weeks. The dose was increased to 100 mg/m^2 if no beneficial effect was seen after 2 treatment cycles. The dose was decreased and/or delayed based on hematologic response or evidence of renal toxicity. Patients in the observation arm were allowed by protocol to cross over to VIDAZA if they had increases in bone marrow blasts, decreases in hemoglobin, increases in red cell transfusion requirements, or decreases in platelets, or if they required a platelet transfusion or developed a clinical infection requiring treatment with antibiotics. For purposes of assessing efficacy, the primary endpoint was response rate (as defined in Table 4).

Of the 191 patients included in the study, independent review (adjudicated diagnosis) found that 19 had the diagnosis of AML at baseline. These patients were excluded from the primary analysis of response rate, although they were included in an intent-to-treat (ITT) analysis of all patients randomized. Approximately 55% of the patients randomized to observation crossed over to receive VIDAZA treatment.

Table 3. Baseline Demographics and Disease Characteristics

	VIDAZA (N=99)	Observation (N=92)
Gender (n%)		
Male	72 (72.7)	60 (65.2)
Female	27 (27.3)	32 (34.8)
Race (n%)		
White	93 (93.9)	85 (92.4)
Black	1 (1.0)	1 (1.1)
Hispanic	3 (3.0)	5 (5.4)
Asian/Oriental	2 (2.0)	1 (1.1)
Age (years)		
N	99	91
Mean ± SD	67.3 ± 10.39	68.0 ± 10.23
Range	31 - 92	35 - 88
Adjudicated MDS diagnosis at study entry (n%)		
RA	21 (21.2)	18 (19.6)
RARS	6 (6.1)	5 (5.4)
RAEB	38 (38.4)	39 (42.4)
RAEB-T	16 (16.2)	14 (15.2)
CMMoL	8 (8.1)	7 (7.6)
AML	10 (10.1)	9 (9.8)
Transfusion product used in 3 months before study entry (n%)		
Any transfusion product	70 (70.7)	59 (64.1)
Blood cells, packed human	66 (66.7)	55 (59.8)
Platelets, human blood	15 (15.2)	12 (13.0)
Hetastarch	0(0.0)	1(1.1)
Plasma protein fraction	1(1.0)	0(0.0)
Other	2(2.0)	2(2.2)

Table 4. Response Criteria

		RA	RARS	RAEB	RAEB-T	CMMoL
Complete Response (CR), duration ≥4 weeks	**Marrow**	<5% blasts				
	Peripheral Blood	Normal CBC if abnormal at baseline Absence of blasts in the peripheral circulation				
Partial Response (PR), duration ≥4 weeks	**Marrow**	No marrow requirements		≥50% decrease in blasts Improvement of marrow dyspoiesis		
	Peripheral Blood	≥50% restoration in the deficit from normal levels of baseline white cells, hemoglobin and platelets if abnormal at baseline No blasts in the peripheral circulation For CMMoL, if WBC is elevated at baseline, a ≥75% reduction in the excess count over the upper limit of normal				

The overall response rate (CR + PR) of 15.7% in VIDAZA-treated patients without AML (16.2% for all VIDAZA randomized patients including AML) was statistically significantly higher than the response rate of 0% in the observation group (p<0.0001) (Table 5). The majority of patients who achieved either CR or PR had either 2 or 3 cell line abnormalities at baseline (79%; 11/14) and had elevated bone marrow blasts or were transfusion dependent at baseline. Patients responding to VIDAZA had a decrease in bone marrow blasts percentage, or an increase in platelets, hemoglobin or WBC. Greater than 90% of the responders initially demonstrated these changes by the 5th treatment cycle. All patients who had been transfusion dependent became transfusion independent during PR or CR. The mean and median duration of clinical response of PR or better was estimated as 512 and 330 days, respectively; 75% of the responding patients were still in PR or better at completion of treatment. Response occurred in all MDS subtypes as well as in patients with adjudicated baseline diagnosis of AML.

Table 5. Response Rates

	VIDAZA (N=89)	Observation Before Crossover (N=83)	
Response	**n (%)**	**n (%)**	**P value**
Overall (CR+PR)	14 (15.7)	0 (0.0)	(<0.0001)
Complete (CR)	5 (5.6)	0 (0.0)	(0.06)
Partial (PR)	9 (10.1)	0 (0.0)	--

Patients in the observation group who crossed over to receive VIDAZA treatment (47 patients) had a response rate of 12.8%.

Study 2, a multi-center, open-label, single-arm study of 72 patients with RAEB, RAEB-T, CMMoL, or AML was also carried out. Treatment with subcutaneous VIDAZA resulted in a response rate (CR + PR) of 13.9%, using criteria similar to those described above. The mean and median duration of clinical response of PR or better was estimated as 810 and 430 days, respectively; 80% of the responding patients were still in PR or better at the time of completion of study involvement. In Study 3, another open-label, single-arm study of 48 patients with RAEB, RAEB-T, or AML, treatment with intravenous VIDAZA resulted in a response rate of 18.8%, again using criteria similar to those described above. The mean and median duration of clinical response of PR or better was estimated as 389 and 281 days, respectively; 67% of the responding patients were still in PR or better at the time of completion of treatment. Response occurred in all MDS subtypes as well as in patients with adjudicated baseline diagnosis of AML in both of these studies. VIDAZA dosage regimens in these 2 studies were similar to the regimen used in the controlled study.

Benefit was seen in patients who did not meet the criteria for PR or better, but were considered "improved." About 24% of VIDAZA-treated patients were considered improved, and about 2/3 of those lost transfusion dependence. In the observation group, only 5/83 patients met criteria for improvement; none lost transfusion dependence. In all 3 studies, about 19% of patients met criteria for improvement with a median duration of 195 days.

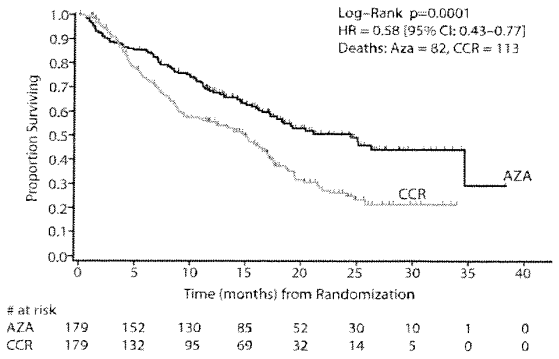
Study 4 was an international, multicenter, open-label, randomized trial in MDS patients with RAEB, RAEB-T or modified CMMoL according to FAB classification and Intermediate-2 and High risk according to IPSS classification. Of the 358 patients enrolled in the study, 179 were randomized to receive azacitidine plus best supportive care (BSC) and 179 were randomized to receive conventional care regimens (CCR) plus BSC (105 to BSC alone, 49 to low dose cytarabine and 25 to chemotherapy with cytarabine and anthracycline). The primary efficacy endpoint was overall survival.

The azacitidine and CCR groups were comparable for baseline parameters. The median age of patients was 69 years (range was 38-88 years), 98% were Caucasian, and 70% were male. At baseline, 95% of the patients were higher risk by FAB classification: RAEB (58%), RAEB-T (34%), and CMMoL (3%). By IPSS classification, 87% were higher risk: Int-2 (41%), High (47%). At baseline, 32% of patients met WHO criteria for AML.

Azacitidine was administered subcutaneously at a dose of 75 mg/m^2 daily for 7 consecutive days every 28 days (which constituted one cycle of therapy). Patients continued treatment until disease progression, relapse after response, or unacceptable toxicity. Azacitidine patients were treated for a median of 9 cycles (range 1 to 39), BSC only patients for a median of 7 cycles (range 1 to 26), low dose cytarabine patients for a median of 4.5 cycles (range 1 to 15), and chemotherapy with cytarabine and anthracycline patients for a median of 1 cycle (range 1 to 3, i.e. induction plus 1 or 2 consolidation cycles).

In the Intent-to-Treat analysis, patients treated with azacitidine demonstrated a statistically significant difference in overall survival as compared to patients treated with CCR (median survival of 24.5 months vs. 15.0 months; stratified log-rank p=0.0001). The hazard ratio describing this treatment effect was 0.58 (95% CI: 0.43, 0.77).

Kaplan-Meier Curve of Time to Death from Any Cause: (Intent-to-Treat Population)



Key: AZA = azacitidine; CCR = conventional care regimens; CI = confidence interval; HR = Hazard Ratio

Azacitidine treatment led to a reduced need for red blood cell transfusions (see Table 6). In patients treated with azacitidine who were RBC transfusion dependent at baseline and became transfusion independent, the median duration of RBC transfusion independence was 13.0 months.

Table 6. Effect of Azacitidine on RBC Transfusions in MDS Patients

Efficacy Parameter	Azacitidine plus BSC (n= 179)	Conventional Care Regimens (n= 179)
Number and percent of patients who were transfusion dependent at baseline who became transfusion independent on treatment[1]	50/111 (45.0%) (95% CI: 35.6%, 54.8%)	13/114 (11.4%) (95% CI: 6.2%, 18.7%)
Number and percent of patients who were transfusion-independent at baseline who became transfusion-dependent on treatment	10/68 (14.7%) (95% CI: 7.3%, 25.4%)	28/65 (43.1%) (95% CI: 30.9%, 56.0%)

[1] A patient was considered RBC transfusion independent during the treatment period if the patient had no RBC transfusions during any 56 consecutive days or more during the treatment period. Otherwise, the patient was considered transfusion dependent.

15 REFERENCES

1. Preventing Occupational Exposures to Antineoplastic and Other Hazardous Drugs in Health Care Settings. NIOSH Alert 2004-165.
2. OSHA Technical Manual, TED 1-0.15A, Section VI: Chapter 2. Controlling Occupational Exposure to Hazardous Drugs. OSHA, 1999. http://www.osha.gov/dts/osta/otm/otm_vi/otm_vi_2.html
3. American Society of Health-System Pharmacists. ASHP guidelines on handling hazardous drugs. Am J Health-Syst Pharm. (2006) 63:1172-1193.
4. Polovich, M., White, J. M., & Kelleher, L.O. (eds.) 2005. Chemotherapy and biotherapy guidelines and recommendations for practice (2nd. ed.) Pittsburgh, PA: Oncology Nursing Society.

16 HOW SUPPLIED/STORAGE AND HANDLING

How Supplied

VIDAZA (azacitidine for injection) is supplied as a lyophilized powder in 100 mg single-use vials packaged in cartons of 1 vial (NDC 59572-102-01).

Storage

Store unreconstituted vials at 25° C (77° F); excursions permitted to 15°-30° C (59°-86° F) (See USP Controlled Room Temperature).

Handling and Disposal

Procedures for proper handling and disposal of anticancer drugs should be applied. Several guidelines on this subject have been published.[1-4] There is no general agreement that all of the procedures recommended in the guidelines are necessary or appropriate.

17 PATIENT COUNSELING INFORMATION

Instruct patients to inform their physician about any underlying liver or renal disease.

Advise women of childbearing potential to avoid becoming pregnant while receiving treatment with VIDAZA. For nursing mothers, a decision should be made whether to discontinue nursing or to discontinue the drug, taking into consideration the importance of the drug to the mother.

Advise men not to father a child while receiving treatment with VIDAZA.

Manufactured for: Celgene Corporation
Summit, NJ 07901

Manufactured by: Ben Venue Laboratories, Inc.
Bedford, OH 44146

Or

Baxter Oncology GmbH
33790 Halle/Westfalen Germany

Or

BSP Pharmaceuticals S.r.l.
04013 Latina Scalo (Lt)
Italy

VIDPI.007 12/12

EXHIBIT C

VOLUME 29 · NUMBER 18 · JUNE 20 2011

JOURNAL OF CLINICAL ONCOLOGY

ORIGINAL REPORT

Phase I Study of Oral Azacitidine in Myelodysplastic Syndromes, Chronic Myelomonocytic Leukemia, and Acute Myeloid Leukemia

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Submitted December 21, 2010; accepted March 22, 2011; published online ahead of print at www.jco.org on May 16, 2011.

Supported by Celgene; editorial support in the preparation of this manuscript from Excerpta Medica, sponsored by Celgene.

Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

Clinical Trials repository link available on JCO.org.

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0732-183X/11/2918-2521/$20.00

DOI: 10.1200/JCO.2010.34.4226

ABSTRACT

Purpose
To determine the maximum-tolerated dose (MTD), safety, pharmacokinetic and pharmacodynamic profiles, and clinical activity of an oral formulation of azacitidine in patients with myelodysplastic syndromes (MDSs), chronic myelomonocytic leukemia (CMML), or acute myeloid leukemia (AML).

Patients and Methods
Patients received 1 cycle of subcutaneous (SC) azacitidine (75 mg/m^2) on the first 7 days of cycle 1, followed by oral azacitidine daily (120 to 600 mg) on the first 7 days of each additional 28-day cycle. Pharmacokinetic and pharmacodynamic profiles were evaluated during cycles 1 and 2. Adverse events and hematologic responses were recorded. Cross-over to SC azacitidine was permitted for nonresponders who received ≥ 6 cycles of oral azacitidine.

Results
Overall, 41 patients received SC and oral azacitidine (MDSs, n = 29; CMML, n = 4; AML, n = 8). Dose-limiting toxicity (grade 3/4 diarrhea) occurred at the 600-mg dose and MTD was 480 mg. Most common grade 3/4 adverse events were diarrhea (12.2%), nausea (7.3%), vomiting (7.3%), febrile neutropenia (19.5%), and fatigue (9.8%). Azacitidine exposure increased with escalating oral doses. Mean relative oral bioavailability ranged from 6.3% to 20%. Oral and SC azacitidine decreased DNA methylation in blood, with maximum effect at day 15 of each cycle. Hematologic responses occurred in patients with MDSs and CMML. Overall response rate (ie, complete remission, hematologic improvement, or RBC or platelet transfusion independence) was 35% in previously treated patients and 73% in previously untreated patients.

Conclusion
Oral azacitidine was bioavailable and demonstrated biologic and clinical activity in patients with MDSs and CMML.

J Clin Oncol 29:2521-2527. © 2011 by American Society of Clinical Oncology

INTRODUCTION

Azacitidine is a cytidine nucleoside analog with a mechanism of action that involves incorporation into DNA and RNA.[1,2] Data suggest that patients must be exposed to azacitidine over several treatment cycles for optimal therapeutic effect.[3] The requirement for chronic exposure can be explained by drug pharmacokinetics, as azacitidine has a short plasma half-life, and by mechanism of action, as induction of DNA hypomethylation through incorporation into DNA is cell-cycle dependent (S-phase restricted) and DNA remethylation is observed by the end of each treatment cycle.[4]

A treatment regimen facilitating chronic administration may help achieve optimal efficacy outcomes. An oral azacitidine formulation would improve convenience of administration and expand the possibilities of exploring novel maintenance schedules, targeting different malignancies, and testing multiple combinations. A phase 0 trial demonstrated that a single oral azacitidine dose resulted in detectable levels in the blood.[5]

This phase I study sought to identify the maximum-tolerated dose (MTD), dose-limiting toxicities (DLTs), safety, pharmacokinetic and pharmacodynamic profiles, and clinical activity of oral azacitidine in patients with myelodysplastic syndromes (MDSs), chronic myelomonocytic leukemia (CMML), or acute myeloid leukemia (AML).

PATIENTS AND METHODS

The trial was approved by the relevant institutional review boards and ethics committees. All patients gave written informed consent.

Patients

Eligible patients were ≥ 18 years, had an Eastern Cooperative Oncology Group performance status score of 0 to 2, and a diagnosis of MDSs, CMML, or AML according to WHO classification.[6,7] For patients with AML, eligibility was limited to those for whom standard curative measures did not exist or were no longer effective. Exclusion criteria included a diagnosis of acute promyelocytic leukemia, previous treatment with hypomethylating agents within 4 weeks before cycle 1, and anticancer therapy within 21 days before the first dose of study drug, or less than full recovery from any significant toxic effects of prior treatments.

Table 1. Baseline Patient Characteristics (N = 41)

Parameter	No. of Patients	%
Median age, years	70	
Range	31-91	
Sex		
Male	32	78
Female	9	22
MDSs (WHO classification)	29	71
RA/RARS/RCMD	11	27
RAEB-1	12	29
RAEB-2	5	12
MDSs-U	1	2
CMML	4	10
AML	8	20
De novo	4	10
Transformed from MDSs	4	10
IPSS (MDSs patients)*		
Low risk	2	7
Intermediate 1 risk	12	41
Intermediate 2 risk	13	45
High risk	1	3
Not available†	1	3
Hematology		
Median hemoglobin, g/dL	9.3	
Range	6.9-15.1	
Median white blood cell count $\times 10^9$/L	2.4	
Range	0.4-30.2	
Median absolute neutrophil count $\times 10^9$/L	0.8	
Range	0.0-21.7	
Median platelet count $\times 10^9$/L	54.0	
Range	3.0-262.0	
Cytogenetics‡		
Normal chromosomal karyotype	17	49
1 chromosomal abnormality	9	26
2 chromosomal abnormalities	3	9
≥ 3 chromosomal abnormalities	6	17
Prior treatment with hypomethylating agent	16	39
MDSs	13	32
CMML	0	0
AML	3	7

Abbreviations: AML, acute myeloid leukemia; CMML, chronic myelomonocytic leukemia; IPSS, International Prognostic Scoring System; MDSs, myelodysplastic syndromes; MDSs-U, MDSs unclassified; RA, refractory anemia; RAEB, RA with excess blasts; RARS, RA with ringed sideroblasts; RCMD, refractory cytopenias with multilineage dysplasia.

*IPSS score[11] was available for 28 patients with MDSs.

†Patient had a bone marrow transplantation and therefore IPSS risk was not considered applicable.

‡Cytogenetic data were available for 35 patients.

Study Design and Therapy

This open-label, phase I, dose-escalation trial was performed in four participating institutions and evaluated multiple cycles of oral azacitidine administered daily for the first 7 days of a 28-day cycle. The objectives were to determine the MTD, DLTs, and the safety profile of oral azacitidine. Pharmacokinetic and pharmacodynamic profiles of oral and subcutaneous (SC) azacitidine, administered on the same 7-day schedule, were also compared. A secondary objective was to assess the clinical activity of oral azacitidine.

During cycle 1, patients received azacitidine 75 mg/m^2 daily SC for 7 days of a 28-day cycle. During cycle 2 and beyond, patients received oral azacitidine under fasting conditions (ie, no food for 2 hours before and after dosing). The dose of oral azacitidine was escalated following a standard phase I 3 + 3 design. The starting dose was 120 mg and doses were escalated in 60 mg increments up to a dose of 360 mg, followed by 120 mg increments until the MTD was reached. Intrapatient dose escalation was permitted if the dose level to which the patient was escalated was associated with a DLT rate of ≤ 33%. Treatment continued until disease progression, lack of activity, unacceptable toxicity, or patient preference.

The MTD was defined as the highest dose at which no more than 33% of patients experienced a DLT. DLT was defined as: grade ≥ 3 nausea, diarrhea, or vomiting despite adequate/maximal medical intervention; grade ≥ 3 clinically significant nonhematologic toxicity unrelated to underlying disease or intercurrent illness; failure to recover to an absolute neutrophil count (ANC) of higher than 500/μL and/or platelet count of higher than 25,000/μL with hypocellular bone marrow (< 5%) 42 days after starting oral azacitidine (patients with a baseline ANC of ≤ 500/μL and/or platelet count of ≤ 25,000/μL were not evaluable for neutrophil or platelet toxicity); any treatment-related effect resulting in missing ≥ 3 oral azacitidine doses in the 7-day treatment period; or any treatment-related nonhematologic toxicity delaying initiation of the second oral azacitidine cycle by longer than 14 days. Only DLTs that occurred during the first oral azacitidine cycle were considered in determining the MTD. Adverse events were graded using the National Cancer Institute Common Toxicity Criteria for Adverse Events version 3.0.

Table 2. Incidence of Adverse Events According to Severity in ≥ 20% of Patients Treated With Oral Azacitidine (n = 41)

System Organ Class Preferred Term (MedDRA 10.1)	CTCAE Grade 1		2		3		4		Total	
	No.	%	No.	%	No.	%	No.	%	No.	%
Diarrhea	10	24.4	12	29.3	4	9.8	1	2.4	27	65.9
Nausea	8	19.5	10	24.4	3	7.3	0		21	51.2
Constipation	9	22.0	7	17.1	0		0		16	39.0
Vomiting	4	9.8	6	14.6	3	7.3	0		13	31.7
Abdominal pain	6	14.6	4	9.8	0		0		10	24.4
Headache	7	17.1	5	12.2	1	2.4	0		13	31.7
Fatigue	6	14.6	2	4.9	4	9.8	0		12	29.3
Peripheral edema	11	26.8	1	2.4	0		0		12	29.3
Fever	6	14.6	2	4.9	2	4.9	0		10	24.4
Cough	7	17.1	1	2.4	2	4.9	0		10	24.4
Contusion	9	22.0	0		0		0		9	22.0
Dizziness	5	12.2	3	7.3	0		0		8	19.5
Febrile neutropenia	0		0		8	19.5	0		8	19.5

NOTE. This Table includes all adverse events which started during any dosing cycle at which oral azacitidine was administered. Percentages are based on the number of patients who received at least one dose of oral azacitidine. Multiple reports of the same preferred term from a patient are counted only once, using the maximum CTCAE grade.

Abbreviations: CTCAE, National Cancer Institute Common Toxicity Criteria for Adverse Events; MedDRA, Medical Dictionary for Regulatory Activities.

Pharmacokinetic Analysis

Plasma and urine pharmacokinetic evaluation of azacitidine was performed on days 1 and 7 in cycles 1 and 2. Samples were collected up to 8 hours after administration and analyzed using a validated high-performance liquid chromatography/tandem mass spectrometric method. Parameters calculated using noncompartmental method, included maximum observed plasma concentration (C_{max}), time of maximum observed plasma concentration (T_{max}), area under the plasma concentration–time curve from zero to infinity (AUC_{inf}), apparent total clearance (CL/F), relative oral bioavailability (F), and apparent volume of distribution (Vd/F).

Pharmacodynamic Analysis

DNA methylation levels were measured to determine DNA hypomethylating activity of azacitidine when administered SC or orally. Whole blood was collected at baseline and before drug administration on days 3, 8, 15, and 22 of cycle 1, and days 1, 3, 8, 15, 22, and 28 of cycle 2. Genomic DNA was purified from each whole blood sample using the PAXgene Blood DNA System (Qiagen; Valencia, CA). DNA methylation was analyzed using the Infinium Human Methylation27 BeadArray (Illumina; San Diego, CA). In cycle 1, DNA methylation data were generated from blood samples of 15 patients. For 10 of these patients, data were also generated in cycle 2. A methylation ratio, or beta value, for each locus per sample was calculated as methylated signal/(methylated + unmethylated signal). Those with detection $P \leq .05$ were considered high-quality measures. Samples with more than 25,200 high-quality beta values and 26,304 autosomal loci with high-quality beta values in at least half of the samples were used for analyses. The low-quality beta values were reimputed using the pamr.knnimpute function from the R package pamr.[8] Wilcoxon signed-rank tests were performed to identify loci with significant methylation differences at each post-treatment time point versus baseline; $P < .01$ was considered statistically significant. All statistical analyses were carried out in R (R Foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org).

Clinical Activity

Data for clinical activity were evaluated using International Working Group (IWG) 2006 criteria, with modifications as described below, for patients with MDSs or CMML[9] and IWG 2003 criteria for patients with AML.[10] Complete remission (CR), hematologic improvement (HI), and RBC and platelet transfusion independence (TI) were evaluated for patients with MDSs or CMML. Bone marrow CR (mCR) was also evaluated but not included in the overall response rate. RBC transfusion dependence at baseline was defined as ≥ 4 RBC units in the 56 days before cycle 1. Platelet transfusion dependence

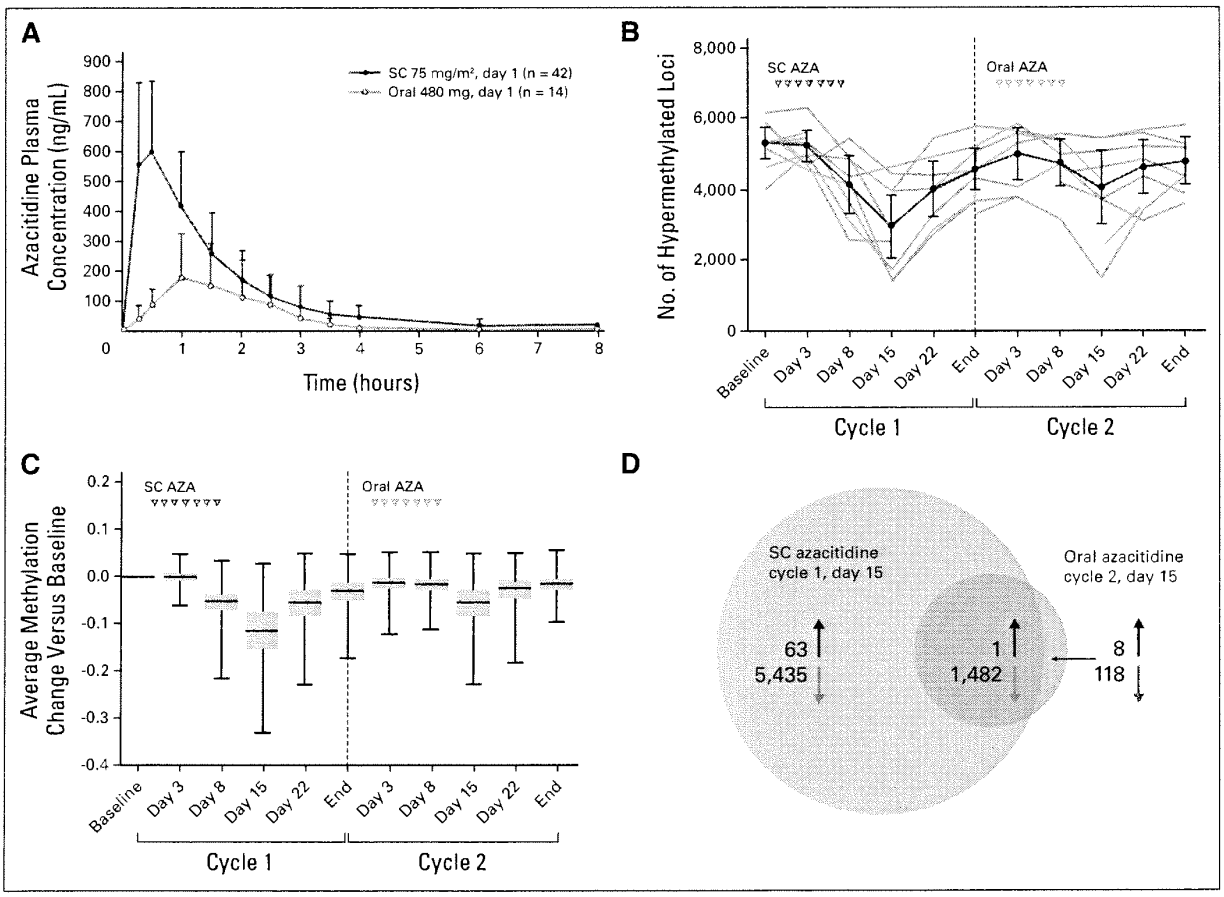

Fig 1. (A) Mean azacitidine (AZA) plasma concentration versus time profiles following single subcutaneous (SC) or oral administration (linear scale). (B) Pharmacodynamics as measured by plotting the numbers of highly methylated loci (beta ≥ 0.7; ± 95% CI) for 10 patients with DNA methylation data in cycles 1 and 2 (gold lines represent individual patients, blue line represents the average). (C) Change in methylation level during treatment with SC or oral AZA for 5,232 loci highly methylated at baseline (blue box represents the 25th to 75th percentile, horizontal band represents the median, vertical line with bars represents minimum and maximum values). (D) Number of significantly differentially methylated loci on day 15 of cycle 1 (SC azacitidine) and on day 15 of cycle 2 (oral azacitidine). Upward arrows denote hypermethylated loci and downward arrows denote hypomethylated loci.

at baseline was defined as ≥ 2 platelet transfusions in the 56 days before cycle 1 (modification to IWG 2006 criteria). RBC and platelet TI were defined as no transfusions in any 56 consecutive-day period on treatment. Patients who achieved ≥ 50% reduction in platelet transfusion requirement, but not platelet TI, in any 56 consecutive-day period on treatment were counted as having achieved HI platelet (HI-P; modification to IWG 2006 criteria). Patients RBC transfusion dependent at baseline achieving a ≥ 50% reduction in RBC transfusion requirement in any 56-consecutive day period and patients not RBC transfusion dependent at baseline, but who achieved a 1.5 g/dL increase in hemoglobin in any 56-consecutive day period on treatment were considered to have achieved HI erythroid (HI-E; modification to IWG 2006 criteria). All patients who received ≥ 1 cycle of oral azacitidine were included in the response analysis. The cutoff date for data in this article was August 19, 2010.

RESULTS

Patient Characteristics

Forty-five patients were treated on a 7-day once-daily schedule. Four patients received the first cycle of SC azacitidine only; three discontinued due to progressive disease (including one death), and one withdrew consent. Baseline characteristics for the remaining 41 patients who received oral azacitidine are presented in Table 1.[11] Cytogenetic data were available at baseline for 35 of 41 patients treated with oral azacitidine; nearly half of the patients had normal karyotype, approximately 25% had a single abnormality, and nearly 20% had a complex karyotype (≥ 3 chromosomal abnormalities). Overall, 16 (39%) of 41 patients had received prior hypomethylating therapy.

Dose Escalation of Oral Azacitidine

No DLTs were observed at dose levels up to 480 mg. DLT was observed at the 600 mg dose, with two (66.7%) of three patients experiencing severe diarrhea, despite adequate medical intervention (grade 3 in one patient and grade 4 in the other). Per protocol, the MTD was exceeded and the previous dose level of 480 mg was determined to be the MTD.

Safety Profile

Table 2 shows the incidence of AEs (any grade) that occurred in ≥ 20% of patients treated with oral azacitidine. The most frequently observed AEs were gastrointestinal disorders, headache, fatigue, and peripheral edema. Other commonly occurring AEs included fever, cough, contusion, dizziness, and febrile neutropenia. Grade 3/4 nausea and grade 3/4 vomiting were each observed in 7% of patients. Grade 3 fatigue was observed in 10% of patients. Diarrhea occurred at grade 3 severity in 10% of patients and grade 4 severity in 2%. Grade 3 febrile neutropenia was observed in eight patients (20%), with four of those having an ANC of ≤ 500/μL at baseline.

Of the 41 patients who received oral azacitidine, 33 terminated from the study as of the date of data analysis, with 17 discontinuing before completing 6 cycles of oral therapy. Reasons for discontinuation included disease progression/treatment failure (n = 10), investigator decision primarily due to absence of observed benefit/response (n = 15), withdrawal of consent (n = 4), AEs (n = 3), and decision to pursue hematopoietic stem-cell transplantation (n = 1). There were three deaths within 28 days of last dose of study drug due to multiple organ failure (n = 1), gastrointestinal hemorrhage (n = 1), and pneumonia plus urinary tract infection (n = 1). No deaths were attributed to study drug. Eight patients remained on the study at the time of data analysis, having each received between 14 and 32 treatment cycles.

Pharmacokinetic Characteristics of Azacitidine

High interpatient variability was noted for all pharmacokinetic parameters. Azacitidine was rapidly absorbed after SC (n = 42) and oral (n = 36) administration, reaching C_{max} within 0.5 hours (range, 0.2 to 1.1 hours) and 1.0 hours (range, 0.3 to 3.6 hours) postdose, respectively. Concentration versus time profiles decreased in a pseudobiphasic manner (Fig 1A). The mean elimination half-life was 1.6 ± 0.7 hours for SC and 0.62 ± 0.25 hours for oral azacitidine. Exposure after single oral administration generally increased with dose (Table 3). For the seven oral dose levels, the mean relative azacitidine oral bioavailability (F) ranged from 6.3% to 20%. The MTD had a mean relative bioavailability of 13% ± 9%. CL/F exceeded hepatic blood flow, indicating extrahepatic metabolism, and Vd/F was greater than total body water, suggesting extensive tissue distribution. The amount of azacitidine recovered in urine relative to dose was small (< 2%) for

Table 3. Day 1 Plasma Pharmacokinetics Parameters After Single Subcutaneous or Oral Azacitidine Administration

Dose	No. of Patients	AUC_{inf} (ng × h/mL) Mean	SD	%CV	CL/F (L/h) Mean	SD	%CV	C_{max} (ng/mL) Mean	SD	%CV	T_{max} (h) Median	Range	Vd/F (L) Mean	SD	%CV	F (%) Mean	SD	Relative Oral Bioavailability
Subcutaneous, 75 mg/m^2	42	1,020	440	43*	175	128	73*	650	250	39	0.50	0.2-1.1	410	410	101*	NA		
Oral, mg																		
120	4	62	43	70	4,100	4,860	118	38	24	64	1.48	1.0-2.0	2,930	3,810	130	8.1	5.6	69
180	3	112	64	58	2,330	1,890	81	72	36	50	1.50	1.0-1.5	1,700	1,580	93	6.3	2.3	37
240	3	463	221	48	598	258	43	215	102	47	1.00	1.0-1.5	814	421	52	20.0	9.6	48
300	5	282	88	31	1,180	487	41	144	13	9.2	1.48	1.0-2.0	1,090	626	57	11.5	2.6	23
360	5	311	141	45	1,360	573	42	195	79	40	1.00	0.5-3.6	947	251	27	12.8	2.4	19
480	14	362	253	70	2,140	1,620	76	211	140	66	1.00	0.3-2.5	2,010	1,910	95	12.8	9.4	74†
600	2	502	100	20	1,220	244	20	253	29	12	1.50	1.0-2.0	1,580	1,410	89	14.9	0.8	5

Abbreviations: AUC_{inf}, area under the plasma concentration–time curve from time zero to infinity; CL/F, apparent total clearance; C_{max}, maximum observed plasma concentration; F, relative oral bioavailability; NA, not applicable; T_{max}, time of maximum observed plasma concentration; Vd/F, apparent volume of distribution.
*n = 40.
†n = 13.

SC and oral administration, suggesting that nonrenal elimination is the predominant pathway for clearance. Results after multiple doses were similar to those obtained after a single dose for both administration routes (data not shown). There was no evidence of azacitidine accumulation.

Pharmacodynamics of Azacitidine: Effect on DNA Methylation

DNA methylation was evaluated during cycles 1 and 2 in 10 patients treated with oral azacitidine. The numbers of highly methylated loci were calculated at each time point by averaging across patients the number of loci with methylation ratios ≥ 0.7 (Fig 1B). These numbers decreased after SC and oral administration, with maximal effects at day 15 of each cycle. The reduction in levels of highly methylated loci was not maintained throughout the entire cycle and returned to near-baseline levels by the end of each cycle. SC azacitidine decreased a greater number of loci in comparison to oral azacitidine. The changes in methylation level from baseline across patients for the 5,232 highly methylated loci (average methylation ratio at baseline ≥ 0.7) are represented as box plots (Fig 1C). As with the analysis of total numbers of highly methylated loci, the median DNA methylation of these loci was reduced by 0.115 on day 15 of cycle 1 (SC azacitidine) and 0.055 on day 15 of cycle 2 (oral azacitidine), and returned to baseline levels at the end of each cycle.

Differentially methylated loci at each post-treatment time point compared with baseline were identified in cycles 1 and 2, with the maximum number observed on day 15 of each cycle; 6,981 loci were differentially methylated (6,917 hypomethylated) on day 15 of cycle 1 (SC azacitidine) and 1,609 loci were differentially methylated (1,600 hypomethylated) on day 15 of cycle 2 (oral azacitidine; $P < .01$). In total, 1,482 loci were significantly hypomethylated by both SC and oral azacitidine (Fig 1D), representing 92.6% of all loci significantly hypomethylated by oral azacitidine treatment. These data demonstrate comparable biologic activity with SC and oral azacitidine, albeit to a lesser extent with oral azacitidine.

Clinical Activity of Oral Azacitidine

The median number of oral azacitidine cycles administered to patients with MDSs, CMML, and AML was 6 (range, 1 to 32+), 12.5+ (range, 3 to 28+), and 4.5 (range, 1 to 15), respectively. Treatment duration is summarized in Figure 2. The number of patients from the MDSs, CMML, and AML groups who remained on the study at the

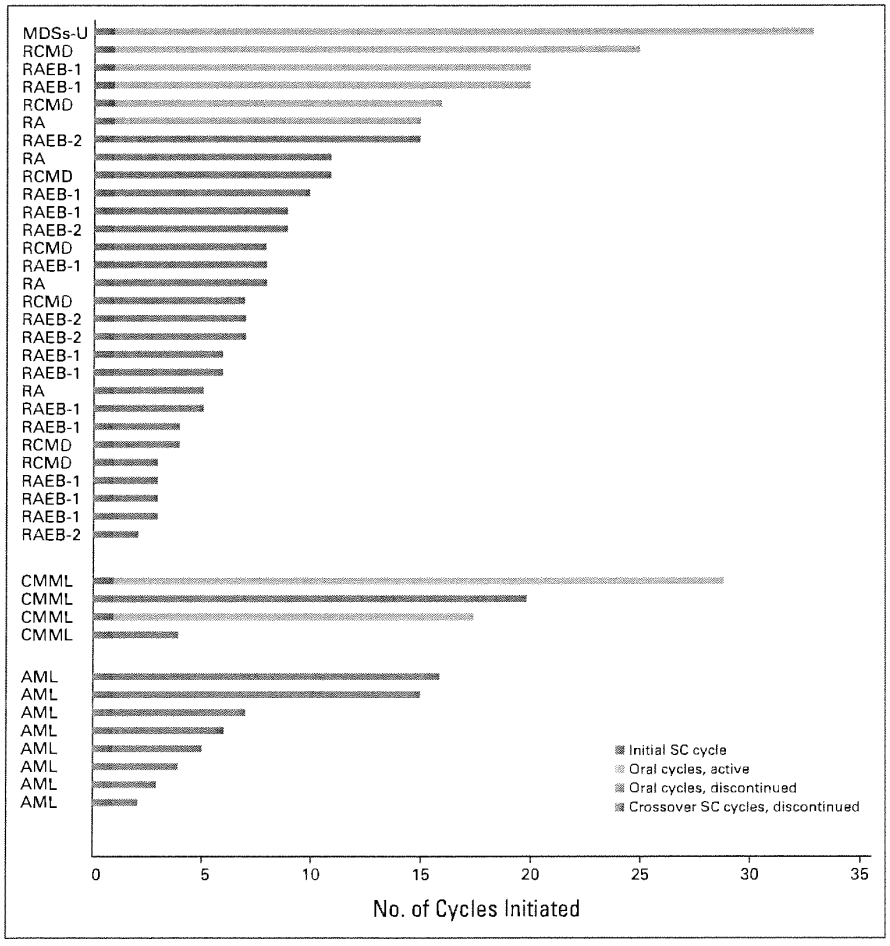

Fig 2. Treatment duration for the 41 patients treated with oral azacitidine (AZA). AML, acute myeloid leukemia; CMML, chronic myelomonocytic leukemia; MDSs-U, myelodysplastic syndromes-unclassified; RA, refractory anemia; RCMD, refractory cytopenias with multilineage dysplasia; RAEB, RA with excess blasts; SC, subcutaneous.

Table 4. Response in Myelodysplastic Syndromes and Chronic Myelomonocytic Leukemia Patients

Response	Previously Treated Patients[a]			First-Line Treatment			Duration of Response: Range (days)
	Responders	Evaluable Patients	%	Responders	Evaluable Patients	%	
Overall response[b]	6	17	35	11	15	73	30-483[c]
CR[d]	0	17	0	6	15	40	30-152
Any HI[e]	6	16	38	5	9	56	56-483[c]
HI-E	3	10	30	2	4	50	56-483[c]
HI-N	0	10	0	2	7	29	82-321[c]
HI-P	5	14	36	2	6	33	58-351[c]
TI	0	5	0	1	3	33	76
Red blood cell	0	3	0	1	3	33	76
Platelet	0	4	0	0			NA
mCR[e,f]	6	9	67	2	6	33	63-422[g]

NOTE. At any cycle of azacitidine, International Working Group 2006 criteria were used with modifications as described in the Patients and Methods section.
Abbreviations: CR, complete remission; E, erythroid; HI, hematologic improvement; mCR, bone marrow complete remission; N, neutrophil; NA, not applicable; P, platelet; TI, transfusion independence.
[a]Includes erythropoiesis-stimulating agents, chemotherapy, hypomethylating agents, and investigational and/or other agents.
[b]Overall response rate does not include patients achieving mCR only.
[c]One or more responses, including that at upper limit of range, are ongoing. Data were censored as of last visit entered into the clinical database.
[d]Patients achieving CR were not included in any other categories.
[e]One patient with mCR in the previously treated group also achieved HI (both HI-E and HI-P). Two patients with mCR in the first-line treatment group also achieved HI (one patient with HI-P and one patient with both HI-E and HI-N). These patients have been included in both the mCR and HI categories.
[f]In the eight patients who achieved mCR, the response began in cycle 1 of subcutaneous (SC) dosing (n = 4) or very early in cycle 2 of oral dosing (n = 4). Therefore, the contribution of a single SC azacitidine cycle to the induction of these responses is likely relevant.
[g]Bone marrow aspirates were not required after 6 cycles of oral azacitidine treatment, therefore follow-up data were not available to confirm upper limit of duration. Data were censored as of last visit entered into the clinical database.

time of the analysis was 6, 2, and 0, respectively. Response and duration of response data are summarized in Table 4. In the 17 previously treated patients with MDSs and CMML, the overall response rate was 35%, without including patients who only achieved mCR; if those patients were included the response rate would be 65%. In the 15 patients with MDSs and CMML receiving first-line treatment, the overall response rate was 73% and in this group no patients achieved mCR only. Longest duration of response to date was 483 days overall. In one patient who achieved a CR, the response began before oral dosing and ended in cycle 2, thus was likely attributable to the single cycle of SC azacitidine.

No responses were observed in patients with AML. Two patients with AML (25%) had stable disease for 14 and 15 cycles, respectively, and five patients with AML (63%) received ≥ 4 oral azacitidine cycles.

DISCUSSION

An oral azacitidine formulation may bring advantages for patients (ease of administration), society (health care cost implications), and disease treatment (extended administration), provided that clinical activity and safety are similar to SC/intravenous azacitidine. This phase I trial demonstrated that oral azacitidine is associated with minimal adverse effects at doses lower than 600 mg. The MTD was 480 mg on a 7-day of 28 days treatment schedule. The 600 mg dose was associated with early onset of severe diarrhea in two of three patients. Diarrhea in patients taking oral azacitidine doses lower than 600 mg was self-limiting and manageable by treatment and/or prophylaxis with antidiarrheal agents and/or dose reduction. Azacitidine, along with one or more ingredients used in its formulation, may contribute to the diarrhea observed because it was a common adverse event at most dose levels tested. Gastrointestinal disturbances may have been exacerbated by the requirement to ingest oral azacitidine in a fasting state. Whether oral azacitidine administration with food can reduce gastrointestinal toxicity will be evaluated in ongoing studies. Grade 3 and 4 AEs consisted primarily of febrile neutropenia, gastrointestinal disturbances, and fatigue. Of the eight patients who experienced grade 3 febrile neutropenia, four entered the study with a baseline ANC of $\leq 500/\mu L$.

After oral administration, maximum azacitidine plasma concentrations were achieved rapidly (within 1 hour), suggesting that absorption occurs from the proximal gastrointestinal tract. Azacitidine exposure increased with increasing oral doses, and the mean relative oral bioavailability ranged from 6.3% to 20%. After multiple doses, there was no evidence of azacitidine accumulation, and no apparent decline in absorption was seen between days 1 and 7. Azacitidine clearance was hepatic and extrahepatic, with little evidence of renal clearance.

Kinetics of the change in DNA methylation levels after SC and oral azacitidine were similar, with maximum hypomethylation achieved on day 15, and methylation levels returned to near-baseline values by the end of each cycle. This pattern has been observed in other azacitidine studies.[3,12] At the dosing schedule employed in this study, oral azacitidine affected fewer loci than SC azacitidine; however, 1,482 loci were identified as commonly hypomethylated by both azacitidine formulations.

Significant responses were observed in patients with MDSs and CMML, indicating that oral azacitidine has clinical activity in these settings. Although all patients received an initial cycle of SC azacitidine, which may have contributed to the clinical activity observed, it has been reported that only half of the total hematologic responses to SC azacitidine manifest within 2 cycles.[13] Continued treatment with oral azacitidine following the single cycle of SC azacitidine is therefore likely to be associated with the development and/or maintenance of clinical responses observed in this study.

Results from a study investigating alternative SC azacitidine dosing schedules in lower-risk patients with MDSs suggested that for all

dosing regimens tested, continued azacitidine treatment may be beneficial.[14] The short plasma half-life of azacitidine, S-phase restricted incorporation into DNA, and rapid remethylation of DNA, are contributing factors to the importance of chronic exposure to the drug. It is therefore likely that extended schedules of oral administration will positively affect clinical activity of azacitidine. A follow-up trial has been initiated to investigate the efficacy of such extended schedules.

In conclusion, the MTD for oral azacitidine administered daily for 7 days of a 28-day cycle was determined to be 480 mg, and oral azacitidine is bioavailable and biologically active. Clinical responses were reported in 35% of previously treated patients with MDSs and CMML, and in 73% of patients who received oral azacitidine as first-line therapy. Lower drug exposure and DNA hypomethylation seen with oral azacitidine relative to SC azacitidine provide the rationale for further study of more frequent dosing and extended schedules of oral azacitidine in MDSs, CMML, and AML. While these results show promise for an oral formulation of azacitidine, they are preliminary data and need further research so that these positive early findings can be confirmed in larger numbers of patients.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

Employment or Leadership Position: Tao Shi, Celgene (C); Kyle J. MacBeth, Celgene (C); Eric Laille, Celgene (C); Heidi Giordano, Celgene (C); Barry Skikne, Celgene (C) **Consultant or Advisory Role:** Guillermo Garcia-Manero, Celgene (C); Steven D. Gore, Celgene (C) **Stock Ownership:** Steven D. Gore, Celgene; Renee Ward, Celgene; Tao Shi, Celgene; Kyle J. MacBeth, Celgene; Eric Laille, Celgene; Heidi Giordano, Celgene; Barry Skikne, Celgene **Honoraria:** Christopher Cogle, Celgene **Research Funding:** Guillermo Garcia-Manero, Celgene; Steven D. Gore, Celgene; Christopher Cogle, Celgene; Kyle J. MacBeth, Celgene; Hagop Kantarjian, Celgene; Barry Skikne, Celgene **Expert Testimony:** None **Other Remuneration:** None

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Final approval of manuscript: All authors

REFERENCES

1. Hollenbach PW, Nguyen AN, Brady H, et al: A comparison of azacitidine and decitabine activities in acute myeloid leukemia cell lines. PLoS One 5:e9001, 2010
2. D'Alò F, Voso MT, Leone G: Profile of azacitidine. Therapy 2:717-731, 2005
3. Gore SD, Baylin S, Sugar E, et al: Combined DNA methyltransferase and histone deacetylase inhibition in the treatment of myeloid neoplasms. Cancer Res 66:6361-6369, 2006
4. Streseman C, Bokelmann I, Mahlknecht U, et al: Azacytidine causes complex DNA methylation responses in myeloid leukemia. Mol Cancer Ther 7:2998-3005, 2008
5. Garcia-Manero G, Stoltz ML, Ward MR, et al: A pilot pharmacokinetic study of oral azacitidine. Leukemia 22:1680-1684, 2008
6. Vardiman JW, Harris NL, Brunning RD: The World Health Organization (WHO) classification of the myeloid neoplasms. Blood 100:2292-2302, 2002
7. Vardiman JW, Thiele J, Arber DA, et al: The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: Rationale and important changes. Blood 114:937-951, 2009
8. Troyanskaya O, Cantor M, Sherlock G, et al: Missing value estimation methods for DNA microarrays. Bioinformatics 17:520-525, 2001
9. Cheson BD, Greenberg PL, Bennett JM, et al: Clinical application and proposal for modification of the International Working Group (IWG) response criteria in myelodysplasia. Blood 108:419-425, 2006
10. Cheson BD, Bennett JM, Kopecky KJ, et al: Revised recommendations of the International Working Group for Diagnosis, Standardization of Response Criteria, Treatment Outcomes, and Reporting Standards for Therapeutic Trials in Acute Myeloid Leukemia. J Clin Oncol 21:4642-4649, 2003
11. Greenberg P, Cox C, LeBeau MM, et al: International scoring system for evaluating prognosis in myelodysplastic syndromes. Blood 89:2079-2088, 1997
12. Soriano AO, Yang H, Faderl S, et al: Safety and clinical activity of the combination of 5-azacytidine, valproic acid, and all-trans retinoic acid in acute myeloid leukemia and myelodysplastic syndrome. Blood 110:2302-2308, 2007
13. Silverman LR, Fenaux P, Mufti GJ, et al: Continued azacitidine therapy beyond time of first response improves quality of response in patients with higher-risk myelodysplastic syndromes. Cancer [Epub ahead of print on January 10, 2011]
14. Lyons RM, Cosgriff TM, Modi SS, et al: Hematologic response to three alternative dosing schedules of azacitidine in patients with myelodysplastic syndromes. J Clin Oncol 27:1850-1856, 2009

EXHIBIT D

Blood (ASH Annual Meeting Abstracts) 2012 120: Abstract **424**

Oral Sessions

633. Myelodysplastic Syndromes: Updates on Therapy and Prognosis

Safety and Efficacy of Oral Azacitidine (CC-486) Administered in Extended Treatment Schedules to Patients with Lower-Risk Myelodysplastic Syndromes

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Abstract 424

Background: Azacitidine for injection has been shown to prolong overall survival in patients (pts) with higher-risk myelodysplastic syndromes (MDS) compared with conventional care regimens (CCR) (*Lancet Oncol*, 2009). An oral formulation of azacitidine (CC-486) is in development. Oral azacitidine may maximize convenience, eliminate injection-site reactions, and if administered in extended dosing schedules, may enhance and prolong the therapeutic effects of azacitidine. Oral azacitidine administered once-daily (QD) for 7 days (d) of repeated 28d cycles has been shown to be bioavailable, biologically and clinically active, and well-tolerated in pts with MDS and acute myeloid leukemia (Garcia-Manero, *J Clin Oncol*, 2011). Preliminary evidence suggests that

extending oral azacitidine dosing to 14d or 21d of the 28d cycle may enhance pharmacodynamic and epigenetic activity (Laille, *Leuk Res*, 2011).

Purpose: To evaluate hematologic response and safety associated with extended dosing regimens of oral azacitidine in pts with lower-risk MDS.

Methods: This ongoing, multicenter, phase 1 study, enrolled pts with lower-risk (IPSS Low or INT-1) MDS who were RBC transfusion dependent (TD) and/or thrombocytopenic (average platelet count ≤50,000 within 56d prior to the first dose) at baseline. Pts were sequentially assigned to receive oral azacitidine 300mg QD for either 14d or 21d of repeated 28d cycles. Hematologic assessments were made every 2 weeks. Hematologic response was assessed using IWG 2006 criteria (Cheson, *Blood*, 2006). Adverse events (AEs) were graded using NCI-CTCAE version 3.0.

Results: At data cut-off (May 18, 2012), 53 pts with lower-risk MDS had enrolled (300mg oral azacitidine QDx14d, n=26; QDx21d, n=27). Demographic and disease characteristics at baseline were similar in the 14d and 21d treatment cohorts (**Table 1**). Median (range) hematology counts at baseline were Hgb 8.7 g/L (6.0–13.0), ANC 1.6×10^9/L (0–30.3), and platelets 56.0×10^9/L (6.0–564.0). At study entry, 40% of pts had received no prior MDS treatment (except transfusions), 45% had received erythropoiesis-stimulating agents, and 15% had received WBC growth factors. The number of oral azacitidine treatment cycles received ranged from 1 to 12 (median numbers of oral azacitidine cycles were 6 in the QDx14d and 4 in the QDx21d cohorts). Four pts in the 21d cohort and 1 pt in the 14d cohort received reduced oral azacitidine doses (200mg QD). Overall, 10 pts discontinued the study, including 6 pts (3 pts in each cohort) who discontinued due to AEs that may have been treatment-related (gastrointestinal [n=2] or intracranial [n=1] hemorrhage, febrile neutropenia [n=1], pneumonia [n=1], thrombocytopenia [n=1]). Overall response rates (ORR), which included complete (CR) and partial remission (PR), any hematologic improvement (HI), and transfusion independence (TI), ranged from 38.5% in the QDx14d cohort to 29.6% in the QDx21d cohort, and RBC TI was achieved by 47% and 33%, respectively, of pts who were RBC TD at baseline (**Table 2**). For pts who received at least 4 cycles of oral azacitidine (14d, n=19; 21d, n=14), ORR was 47.4% in the 14d and 50.0% in the 21d cohorts, and RBC TI rates in RBC TD pts (n=16) were 67% in the 14d and 57% in the 21d cohorts. The most frequent (≥5%) grade 3/4 hematologic AEs in the QDx14d cohort were anemia (11.5%), thrombocytopenia (11.5%), and neutropenia (7.7%); and in the QDx21d cohort were neutropenia (14.8%), anemia (7.4%), and febrile neutropenia (7.4%). Most frequent grade 3/4 non-hematologic AEs were gastrointestinal, including vomiting (7.7%) in the QDx14d cohort, and diarrhea (11.1%) and vomiting (7.4%) in the QDx21d cohort.

Conclusions: Oral azacitidine 300mg QD administered in extended dosing schedules of 14d or 21d of repeated 28d cycles was effective and well-tolerated in these pts with lower-risk MDS. Beside hematologic AEs, the most frequently observed AEs with oral azacitidine were gastrointestinal and were manageable. Efficacy and safety outcomes with 300mg QD oral azacitidine were generally comparable between the 14d and 21d extended dosing regimens. Based on these data, oral azacitidine administered once-daily

in extended dosing schedules is active and well-tolerated and warrants further investigation in randomized, controlled trials.

Table 1. Patient Demographic and Disesse Characteristics at Baseline

Table 1. Patient Demographic and Disease Characteristics at Baseline

Characteristic	14-day QD (n=26)	21-day QD (n=27)
Age, years Median (range)	73.0 (70 – 77)	70.0 (60 – 76)
RBC transfusion dependent,* n (%)	15 (58)	15 (56)
MDS WHO classification, n (%)		
RA/RARS	8 (31)	9 (33)
RCMD/RCMD-RS	9 (35)	7 (26)
RAEB-1	4 (15)	4 (15)
RAEB-2	0	1 (4)
MDS-U	2 (8)	3 (11)
Del (5q)	1 (4)	1 (4)
IPSS risk classification, n (%)		
Low	6 (23)	9 (33)
Intermediate-1	20 (77)	18 (67)
Cytogenetics, n (%)		
Normal/diploid	11 (42)	15 (56)
≥1 Abnormality	11 (42)	9 (33)
Indeterminate	4 (15)	3 (11)
Prior treatment, n (%)		
Erythropoiesis-stimulating agents	14 (54)	10 (37)
WBC growth factors	4 (15)	4 (15)
Other	7 (27)	5 (19)
None†	9 (35)	12 (44)

*RBC transfusion dependence at baseline was defined as receipt of ≥4 units of packed RBC within 56 days of the first dose of oral azacitidine.
†Other than transfusions.

Table 2. Hematologic Response and Transfusion Independence with Oral Azacitidine 300mg QD in Extended Dosing Schedules (124 and 21d) in Patients with Lower-risk MDS

Table 2. Hematologic Response and Transfusion Independence with Oral Azacitidine 300mg QD in Extended Dosing Schedules (14d and 21d) in Patients with Lower-risk MDS

Parameter	Treatment Schedule n Responders/N Evaluable* (%)		
	14-day QD (n=26)	21-day QD (n=27)	Total (N=53)
Overall Response*† (CR, PR, any HI, TI)	10/26 (38.5)	8/27 (29.6)	18/53 (34.0)
Any HI	6/26 (23.1)	7/27 (25.9)	13/53 (24.5)
HI – E	4/23 (17.4)	6/25 (24.0)	10/48 (20.8)
HI – P	4/17 (23.5)	2/15 (13.3)	6/32 (18.8)
HI – N	2/10 (20.0)	0/6	2/16 (12.5)
Marrow CR (mCR)	0/7	3/5 (60.0)	3/12 (25.0)
RBC transfusion independence (TI)‡			
Sustained for 56 days	7/15 (46.7)	5/15 (33.3)	12/30 (40.0)
Sustained for 84 days	3/15 (20.0)	4/15 (26.6)	7/30 (23.3)

*IWG 2006 criteria.
†Patients are counted only once for overall response, but may be counted more than once in individual response categories.
‡To be evaluated for TI, pts must have been RBC transfusion dependent (TD) at baseline and been on-study at least 56 days. RBC TD at baseline was defined as receipt of ≥4 units of packed RBC within 56 days of the first dose of oral azacitidine.

Disclosures: Garcia-Manero: *Celgene:* Research Funding, Speakers Bureau. **Gore:** *Celgene Corporation:* Consultancy, Research Funding. **Scott:** *Celgene Corporation:* Honoraria, Research Funding, Speakers Bureau. **Hetzer:** *Celgene Corporation:* Employment, Equity Ownership. **Kumar:** *Celgene Corporation:* Employment, Equity Ownership. **Skikne:** *Celgene:* Employment, Equity Ownership.

Footnotes

* Asterisk with author names denotes non-ASH members.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	Jeffrey B. Etter, *et al.*	Confirmation No.:	5370
Serial No.:	12/466,213	Art Unit:	1673
Filed:	May 14, 2009	Examiner:	Lawrence E. Crane
For:	ORAL FORMULATIONS OF CYTIDINE ANALOGS AND METHODS OF USE THEREOF	Attorney Docket No.: CAM:	9516-847-999 501872-999847

INFORMATION DISCLOSURE STATEMENT UNDER 37 C.F.R. § 1.56

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

In accordance with the duty of disclosure imposed by 37 C.F.R. § 1.56 and §1.97 to inform the Patent and Trademark Office of all references coming to the attention of each individual associated with the filing or prosecution of the subject application, which are or may be material to the patentability of any claim of the application, Attorneys for Applicants hereby direct the Examiner's attention to the references listed on the attached Information Disclosure Statement by Applicant.

Copies of non-patent literature documents 1-3 are submitted herewith. Applicants respectfully request that the Examiner review the listed references and that the references be made of record in the file history of the application. Identification of references listed on the Information Disclosure Statement by Applicant is not to be construed as an admission of Applicants or attorneys for Applicants that such references are available as "prior art" against the subject application.

This Information Disclosure Statement is being filed under 37 C.F.R. §1.97(c) after the period specified in 37 C.F.R §1.97(b), but before the mailing date of any of a final action under

37 C.F.R. §1.113, a notice of allowance under 37 C.F.R. §1.311 or an action that otherwise closes prosecution in the application. Therefore, please apply the $180.00 fee set forth in 37 C.F.R. §1.17(p), and any other charges, or any credits, to Jones Day Deposit Account No. 503013 (referencing 501872-999847).

Respectfully submitted,

Date: February 27, 2014

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PTO/SB/06 (09-11)
Approved for use through 1/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 12/466,213	Filing Date 05/14/2009	☐ To be Mailed

ENTITY: ☒ LARGE ☐ SMALL ☐ MICRO

APPLICATION AS FILED – PART I

	(Column 1)	(Column 2)		
FOR	NUMBER FILED	NUMBER EXTRA	RATE ($)	FEE ($)
☒ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	330
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).			
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))				
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL	330

APPLICATION AS AMENDED – PART II

AMENDMENT	02/27/2014	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 42	Minus	** 91	= 0	X $80 =	0
	Independent (37 CFR 1.16(h))	* 3	Minus	***4	= 0	X $420 =	0
	☐ Application Size Fee (37 CFR 1.16(s))						
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
						TOTAL ADD'L FEE	0

AMENDMENT		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))						
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
						TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

LIE
/TAMMY d. MCBETH BROWN/

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NOTICE OF ALLOWANCE AND FEE(S) DUE

84802 7590 05/28/2014
JONES DAY for Celgene Corporation
222 E. 41ST. STREET
NEW YORK, NY 10017

EXAMINER	
CRANE, LAWRENCE E	
ART UNIT	PAPER NUMBER
1673	

DATE MAILED: 05/28/2014

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/466,213	05/14/2009	Jeffrey B. Etter	9516-847-999/501872-847	5370

TITLE OF INVENTION: ORAL FORMULATIONS OF CYTIDINE ANALOGS AND METHODS OF USE THEREOF

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$960	$0	$0	$960	08/28/2014

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the ENTITY STATUS shown above. If the ENTITY STATUS is shown as SMALL or MICRO, verify whether entitlement to that entity status still applies.

If the ENTITY STATUS is the same as shown above, pay the TOTAL FEE(S) DUE shown above.

If the ENTITY STATUS is changed from that shown above, on PART B - FEE(S) TRANSMITTAL, complete section number 5 titled "Change in Entity Status (from status indicated above)".

For purposes of this notice, small entity fees are 1/2 the amount of undiscounted fees, and micro entity fees are 1/2 the amount of small entity fees.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Utility patents issuing on applications filed on or after Dec. 12, 1980 may require payment of maintenance fees. It is patentee's responsibility to ensure timely payment of maintenance fees when due.

PTOL-85 (Rev. 02/11)

PART B - FEE(S) TRANSMITTAL

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Commissioner for Patents
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Alexandria, Virginia 22313-1450
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INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

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Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission
I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

(Depositor's name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/466,213	05/14/2009	Jeffrey B. Etter	9516-847-999/501872-847	5370

TITLE OF INVENTION: ORAL FORMULATIONS OF CYTIDINE ANALOGS AND METHODS OF USE THEREOF

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$960	$0	$0	$960	08/28/2014

EXAMINER	ART UNIT	CLASS-SUBCLASS
CRANE, LAWRENCE E	1673	514-043000

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).
- ❑ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.
- ❑ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list
(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,
(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.
1 _____
2 _____
3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE (B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent) : ❑ Individual ❑ Corporation or other private group entity ❑ Government

4a. The following fee(s) are submitted:
- ❑ Issue Fee
- ❑ Publication Fee (No small entity discount permitted)
- ❑ Advance Order - # of Copies _____

4b. Payment of Fee(s): (**Please first reapply any previously paid issue fee shown above**)
- ❑ A check is enclosed.
- ❑ Payment by credit card. Form PTO-2038 is attached.
- ❑ The Director is hereby authorized to charge the required fee(s), any deficiency, or credits any overpayment, to Deposit Account Number _____ (enclose an extra copy of this form).

5. **Change in Entity Status** (from status indicated above)
- ❑ Applicant certifying micro entity status. See 37 CFR 1.29 — NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.
- ❑ Applicant asserting small entity status. See 37 CFR 1.27 — NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.
- ❑ Applicant changing to regular undiscounted fee status. — NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature _____ Date _____

Typed or printed name _____ Registration No. _____

PTOL-85 Part B (10-13) Approved for use through 10/31/2013. OMB 0651-0033 U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
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84802 7590 05/28/2014

JONES DAY for Celgene Corporation
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NEW YORK, NY 10017

EXAMINER	
CRANE, LAWRENCE E	

ART UNIT	PAPER NUMBER
1673	

DATE MAILED: 05/28/2014

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(Applications filed on or after May 29, 2000)

The Office has discontinued providing a Patent Term Adjustment (PTA) calculation with the Notice of Allowance.

Section 1(h)(2) of the AIA Technical Corrections Act amended 35 U.S.C. 154(b)(3)(B)(i) to eliminate the requirement that the Office provide a patent term adjustment determination with the notice of allowance. See Revisions to Patent Term Adjustment, 78 Fed. Reg. 19416, 19417 (Apr. 1, 2013). Therefore, the Office is no longer providing an initial patent term adjustment determination with the notice of allowance. The Office will continue to provide a patent term adjustment determination with the Issue Notification Letter that is mailed to applicant approximately three weeks prior to the issue date of the patent, and will include the patent term adjustment on the patent. Any request for reconsideration of the patent term adjustment determination (or reinstatement of patent term adjustment) should follow the process outlined in 37 CFR 1.705.

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

OMB Clearance and PRA Burden Statement for PTOL-85 Part B

The Paperwork Reduction Act (PRA) of 1995 requires Federal agencies to obtain Office of Management and Budget approval before requesting most types of information from the public. When OMB approves an agency request to collect information from the public, OMB (i) provides a valid OMB Control Number and expiration date for the agency to display on the instrument that will be used to collect the information and (ii) requires the agency to inform the public about the OMB Control Number's legal significance in accordance with 5 CFR 1320.5(b).

The information collected by PTOL-85 Part B is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450. Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

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2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
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5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Notice of Allowability	Application No. 12/466,213	Applicant(s) ETTER ET AL.	
	Examiner Lawrence E. Crane	Art Unit 1673	AIA (First Inventor to File) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to *the amendment filed February 27, 2014*.
   ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on_____.
2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.
3. ☒ The allowed claim(s) is/are *1,6-11,13-23,33-37,41-50 and 73-82*. As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov .
4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

   Certified copies:

   a) ☐ All b) ☐ Some *c) ☐ None of the:
   1. ☐ Certified copies of the priority documents have been received.
   2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
   3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____.

   Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).
6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)
2. ☒ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date 20140227
3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material
4. ☒ Interview Summary (PTO-413), Paper No./Mail Date *20140514* .
5. ☒ Examiner's Amendment/Comment
6. ☒ Examiner's Statement of Reasons for Allowance
7. ☐ Other _____.

/Lawrence E. Crane/
Primary Examiner, Art Unit 1673

The present application is being examined under the pre-AIA first to invent provisions.

The Group and/or Art Unit location of your application in the PTO has changed. To aid in correlating any papers for this application, all further correspondence regarding this application should be directed to Group 1600, Art Unit **1673**.

An Examiner's Amendment to the record appears below. Should the changes and/or additions be unacceptable to applicant, an amendment may be filed as provided by 37 C.F.R. §1.312. To ensure consideration of such an amendment, it **MUST** be submitted no later than the payment of the Issue Fee.

In claim **73** at line 1, the term "tablet comprises" was amended to read -- tablet further comprises --.

Authorization for this Examiner's Amendment was given in a telephone interview with Colin Hughes and confirmed by Robert Chang on May 14, 2014.

The following is an Examiner's Statement of Reasons for Allowance:

Examiner has reviewed the previous Office action, the above Examiner's Amendment (necessary to address an issue of inadequate antecedent basis), applicant's response including an extensive and detailed declaration filed under 37 C.F.R. § 1.132 filed February 27, 2014, and an additional or supplemental Information Disclosure Statement filed February 27, 2014 citing references relied upon extensively by the noted declaration. The declarant, Mssr. C. L. Beach, has argued in detail in opposition to examiner's rejections citing prior art, noting that the construction of the rejections of record are inappropriate in view of the prior art cited, and that this conclusion is buttressed in several different ways by the detailed analysis of the instant cited prior art presented in the declaration. Additionally data presented in the declaration, and also in the application, noting the surprisingly high anti-neoplastic medicinal activities of the claimed non-enteric coated pharmaceutical composition, has been identified as unexpected, and therefore said data is also a proper basis to argue in favor of a conclusions opposite to the previously asserted conclusions of obviousness.

Additionally examiner notes that applicant's amendments to the claims together with applicant's arguments made in parallel with the noted declaration have rendered all of the

rejections citing prior art, rejections citing 35 U.S.C. §112, and all objections of record moot. Therefore, all rejections and objections of record have been withdrawn. There remaining no recognized bases for objection to, or rejection of, the claims remaining of record, said claims have been found allowable as presently amended.

Any comments considered necessary by applicant must be submitted no later than the payment of the Issue Fee and, to avoid processing delays, should preferably **accompany** the Issue Fee. Such submissions should be clearly labeled "Comments on Statement of Reasons for Allowance."

Papers related to this application may be submitted to Group 1600 via facsimile transmission(FAX). The transmission of such papers must conform with the notice published in the Official Gazette (1096 OG 30, November 15, 1989). The telephone number for sending a FAX (unofficially) directly to Examiner's computer in Group 1600 is **571-273-0651**. The telephone number for sending an Official FAX to the PTO is 571-273-8300.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Examiner L. E. Crane whose telephone number is **571-272-0651**. The examiner can normally be reached between 9:30 AM and 5:00 PM, Monday through Friday.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Ms. S. Anna Jiang, can be reached on **571-272-0627**.

Any inquiry of a general nature or relating to the status of this application should be directed to the Group 1600 receptionist whose telephone number is **571-272-1600**.

All Post-Allowance Correspondence concerning this application must be mailed to:

BOX ISSUE FEE
COMMISSIONER FOR PATENTS
WASHINGTON, DC 20231

OR you can FAX them to the Office of Patent Publications at 571-273-8300, in order to expedite the handling of such correspondence as amendments under 37 C.F.R. §1.312;

Information Disclosure Statements (IDS's), and formal drawings. Sending Post-Allowance papers to Technology Center 1600 will only cause delays in matching papers with the case.

For information concerning status of correspondence sent after receipt of the Notice of Allowance, please contact the Correspondence Branch at **571-272-4200**. The Notice of Allowance also has an insert containing contact information of other items, including Issue Fees, receipt of formal drawings, and the status of the application.

LECrane:lec
05/15/2013

/Lawrence E. Crane/

Primary Examiner, Art Unit 1673

L. E. Crane
Primary Patent Examiner
Technology Center 1600

Examiner-Initiated Interview Summary	**Application No.** 12/466,213	**Applicant(s)** ETTER ET AL.	
	Examiner Lawrence E. Crane	**Art Unit** 1673	

All participants (applicant, applicant's representative, PTO personnel):

(1) *Lawrence E. Crane*. (3) *Robert Chang*.

(2) *Colin Hughes*. (4)_____.

Date of Interview: *14 May 2014*.

Type: ☒ Telephonic ☐ Video Conference
☐ Personal [copy given to: ☐ applicant ☐ applicant's representative]

Exhibit shown or demonstration conducted: ☐ Yes ☒ No.
If Yes, brief description: _____.

Issues Discussed ☐101 ☒112 ☐102 ☐103 ☐Others
(For each of the checked box(es) above, please describe below the issue and detailed description of the discussion)

Claim(s) discussed: *73*.

Identification of prior art discussed: *None*.

Substance of Interview
(For each issue discussed, provide a detailed description and indicate if agreement was reached. Some topics may include: identification or clarification of a reference or a portion thereof, claim interpretation, proposed amendments, arguments of any applied references etc...)

Applicant was advised that claim 73 lacked antecedent basis but that this could be cured by adding the term "further" before the term "comprises." Applicant agreed and authorized an Examiner's Amendment.

Applicant recordation instructions: It is not necessary for applicant to provide a separate record of the substance of interview.

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

☐ Attachment

/Lawrence E. Crane/ Primary Examiner, Art Unit 1673	

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	12466213	ETTER ET AL.
	Examiner	Art Unit
	LAWRENCE E CRANE	1623

✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
=	Allowed	÷	Restricted	I	Interference	O	Objected

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

CLAIM		DATE								
Final	Original	07/25/2011	02/24/2012	08/25/2013	05/19/2014					
1	1	✓	✓	✓	=					
	2	✓	-	-	-					
	3	✓	✓	✓	-					
	4	✓	✓	✓	-					
	5	✓	✓	✓	-					
2	6	✓	✓	✓	=					
3	7	✓	✓	✓	=					
4	8	✓	✓	✓	=					
5	9	✓	✓	✓	=					
6	10	✓	✓	✓	=					
7	11	✓	✓	✓	=					
	12	✓	✓	✓	-					
8	13	✓	✓	✓	=					
9	14	✓	✓	✓	=					
10	15	✓	✓	✓	=					
11	16	✓	✓	✓	=					
12	17	✓	✓	✓	=					
18	18	✓	✓	✓	=					
19	19	✓	✓	✓	=					
20	20	✓	✓	✓	=					
21	21	✓	✓	✓	=					
22	22	✓	✓	✓	=					
28	23	✓	✓	✓	=					
	24	✓	✓	-	-					
	25	✓	✓	-	-					
	26	✓	✓	-	-					
	27	✓	✓	-	-					
	28	✓	✓	-	-					
	29	✓	✓	-	-					
	30	✓	✓	-	-					
	31	✓	✓	✓	-					
	32	✓	✓	✓	-					
29	33	✓	✓	✓	=					
30	34	✓	✓	✓	=					
31	35	✓	✓	✓	=					
32	36	✓	✓	✓	=					

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	12466213	ETTER ET AL.
	Examiner	Art Unit
	LAWRENCE E CRANE	1623

✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
=	Allowed	÷	Restricted	I	Interference	O	Objected

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

CLAIM		DATE								
Final	Original	07/25/2011	02/24/2012	08/25/2013	05/19/2014					
33	37	✓	✓	✓	=					
	38	✓	✓	✓	-					
	39	✓	✓	✓	-					
	40	✓	✓	✓	-					
34	41	✓	✓	✓	=					
35	42	✓	✓	✓	=					
36	43	✓	✓	✓	=					
37	44	✓	✓	✓	=					
38	45	✓	✓	✓	=					
39	46	✓	✓	✓	=					
40	47	✓	✓	✓	=					
41	48	✓	✓	✓	=					
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43	50	✓	✓	✓	=					
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	56	✓	-	-	-					
	57	✓	✓	✓	-					
	58	✓	✓	-	-					
	59	✓	✓	✓	-					
	60	✓	✓	✓	-					
	61	✓	✓	✓	-					
	62	✓	-	-	-					
	63	✓	-	-	-					
	64	✓	-	-	-					
	65	✓	-	-	-					
	66		✓	-	-					
	67		N	-	-					
	68		✓	-	-					
	69		N	-	-					
	70		✓	-	-					
	71		N	-	-					
	72			✓	-					

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	12466213	ETTER ET AL.
	Examiner	Art Unit
	LAWRENCE E CRANE	1623

✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
=	Allowed	÷	Restricted	I	Interference	O	Objected

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

CLAIM		DATE								
Final	Original	07/25/2011	02/24/2012	08/25/2013	05/19/2014					
13	73				=					
14	74				=					
15	75				=					
16	76				=					
17	77				=					
23	78				=					
24	79				=					
25	80				=					
26	81				=					
27	82				=					

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	12466213	ETTER ET AL.
	Examiner	**Art Unit**
	LAWRENCE E CRANE	1673

CPC							
Symbol						**Type**	**Version**
A61K		9	/	2846		I	2013-01-01
A61K		9	/	2886		I	2013-01-01
A61K		9	/	2013		F	2013-01-01
A61K		31	/	706		I	2013-01-01
A61K		31	/	7068		I	2013-01-01
A61K		9	/	2018		I	2013-01-01
			/				
			/				
			/				
			/				
			/				
			/				
			/				
			/				
			/				

CPC Combination Sets							
Symbol					**Type**	**Set**	**Ranking**	**Version**
			/					
			/					

NONE		Total Claims Allowed:	
(Assistant Examiner)	(Date)	43	
/LAWRENCE E CRANE/ Primary Examiner.Art Unit 1673	05/19/2014	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1 and 28	None

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	12466213	ETTER ET AL.
	Examiner	**Art Unit**
	LAWRENCE E CRANE	1673

US ORIGINAL CLASSIFICATION		INTERNATIONAL CLASSIFICATION												
CLASS	SUBCLASS	CLAIMED					NON-CLAIMED							
514	43	A	0	1	N	43 / 04 (2006.01.01)								
CROSS REFERENCE(S)		A	6	1	K	31 / 70 (2006.01.01)								
		C	0	7	H	19 / 12 (2006.01.01)								
CLASS	SUBCLASS (ONE SUBCLASS PER BLOCK)													
536	28.3													

NONE (Assistant Examiner)	(Date)	**Total Claims Allowed:** 43	
/LAWRENCE E CRANE/ Primary Examiner.Art Unit 1673 (Primary Examiner)	05/19/2014 (Date)	O.G. Print Claim(s) 1 and 28	O.G. Print Figure None

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	12466213	ETTER ET AL.
	Examiner	**Art Unit**
	LAWRENCE E CRANE	1673

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original
1	1	17	77	33	37										
2	6	18	18	34	41										
3	7	19	19	35	42										
4	8	20	20	36	43										
5	9	21	21	37	44										
6	10	22	22	38	45										
7	11	23	78	39	46										
8	13	24	79	40	47										
9	14	25	80	41	48										
10	15	26	81	42	49										
11	16	27	82	43	50										
12	17	28	23												
13	73	29	33												
14	74	30	34												
15	75	31	35												
16	76	32	36												

NONE (Assistant Examiner)	(Date)	**Total Claims Allowed:** 43	
/LAWRENCE E CRANE/ Primary Examiner.Art Unit 1673 (Primary Examiner)	05/19/2014 (Date)	O.G. Print Claim(s) 1 and 28	O.G. Print Figure None

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (01-10)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	12466213
Filing Date	2009-05-14
First Named Inventor	Jeffrey B. Etter
Art Unit	1673
Examiner Name	Lawrence E. Crane
Attorney Docket Number	9516-847-999

U.S.PATENTS Remove

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1					

If you wish to add additional U.S. Patent citation information please click the Add button. Add

U.S.PATENT APPLICATION PUBLICATIONS Remove

Examiner Initial*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1					

If you wish to add additional U.S. Published Application citation information please click the Add button. Add

FOREIGN PATENT DOCUMENTS Remove

Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2] i	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
	1							☐

If you wish to add additional Foreign Patent Document citation information please click the Add button Add

NON-PATENT LITERATURE DOCUMENTS Remove

Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /L.C./

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
page 2 of 2	Application Number	12466213
	Filing Date	2009-05-14
	First Named Inventor	Jeffrey B. Etter
	Art Unit	1673
	Examiner Name	Lawrence E. Crane
	Attorney Docket Number	9516-847-999

	AR5 1	GARCIA-MANERO et al., "Safety and efficacy of oral azacitidine (CC-486) administered in extended treatment schedules to patients with lower-risk myelodysplastic syndromes," Blood, 120:Abstract 424 (2012).	☐
	AS5 2	GARCIA-MANERO et al., "Phase I study; of oral azacitidine in myelodysplastic syndromes, chronic myelomonocytic leukemia, and acute myeloid leukemia," J. Clin. Oncol., 29:2521-2527 (2011).	☐
	AT5 3	Vidaza™ (azacitidine for injection) Prescribing Information, dated December 2012.	☐

If you wish to add additional non-patent literature document citation information please click the Add button **Add**

EXAMINER SIGNATURE

Examiner Signature	/Lawrence Crane/	Date Considered	05/12/2014

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /L.C./

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	12466213	ETTER ET AL.
	Examiner	Art Unit
	LAWRENCE E CRANE	1623

CPC- SEARCHED		
Symbol	Date	Examiner

CPC COMBINATION SETS - SEARCHED		
Symbol	Date	Examiner

US CLASSIFICATION SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES		
Search Notes	Date	Examiner
Classification for restriction purposes - 514/43; 535/28.3	7/25/2011	LEC
File CAPLUS search - see search for strategy	7/25/2011	LEC
Inventor name search - (Etter)	7/25/2011	LEC

INTERFERENCE SEARCH			
US Class/ CPC Symbol	US Subclass / CPC Group	Date	Examiner
514	43	5/19/2014	LEC
536	28.3	5/19/2014	LEC

	/LAWRENCE E CRANE/ Primary Examiner.Art Unit 1623

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: Mail Mail Stop ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
or Fax (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

8480 7590 08/28/2014
JONES DAY for Celgene Corporation
222 E. 41ST. STREET
NEW YORK, NY 10017

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission
I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

(Depositor's name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/466,213	05/14/2009	Jeffrey B. Etter	9516-847-999/501872-847	5370

TITLE OF INVENTION: ORAL FORMULATIONS OF CYTIDINE ANALOGS AND METHODS OF USE THEREOF

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$960	$0	$0	$960	08/28/2014

EXAMINER	ART UNIT	CLASS-SUBCLASS
CRANE, LAWRENCE E	1673	514-043000

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).
- ☐ Change of correspondence address (or Change of Correspondence Address Form PTO/SB/122) attached.
- ☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list
(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,
(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.
1 Jones Day
2
3

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE: Celgene Corporation
(B) RESIDENCE: (CITY and STATE OR COUNTRY): Summit, New Jersey

Please check the appropriate assignee category or categories (will not be printed on the patent): ☐ Individual ☒ Corporation or other private group entity ☐ Government

4a. The following fee(s) are submitted:
- ☒ Issue Fee
- ☐ Publication Fee (No small entity discount permitted)
- ☐ Advance Order - # of Copies .....

4b. Payment of Fee(s): **(Please first reapply any previously paid issue fee shown above)**
- ☐ A check is enclosed.
- ☐ Payment by credit card. Form PTO-2038 is attached.
- ☒ The Director is hereby authorized to charge the required fee(s), any deficiency, or credits any overpayment, to Deposit Account Number 503013 (enclose an extra copy of this form).

5. **Change in Entity Status** (from status indicated above)
- ☐ Applicant certifying micro entity status. See 37 CFR 1.29
  NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.
- ☐ Applicant asserting small entity status. See 37 CFR 1.27
  NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.
- ☐ Applicant changing to regular undiscounted fee status.
  NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature /Colin O. Hughes/ Date August 22, 2014
Typed or printed name Colin O. Hughes (for Yeah-Sil Moon, Reg. No. 52,042) Registration No. 72,172

PTOL-85 Part B (10-13) Approved for use through 10/31/2013. OMB 0651-0033 U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Electronic Patent Application Fee Transmittal

Application Number:	12466213
Filing Date:	14-May-2009
Title of Invention:	ORAL FORMULATIONS OF CYTIDINE ANALOGS AND METHODS OF USE THEREOF
First Named Inventor/Applicant Name:	Jeffrey B. Etter
Filer:	Colin O. Hughes/Carrie Hines
Attorney Docket Number:	9516-847-999/501872-847

Filed as Large Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Utility Appl Issue Fee	1501	1	960	960
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
		Total in USD ($)		**960**

Electronic Acknowledgement Receipt

EFS ID:	19939726
Application Number:	12466213
International Application Number:	
Confirmation Number:	5370
Title of Invention:	ORAL FORMULATIONS OF CYTIDINE ANALOGS AND METHODS OF USE THEREOF
First Named Inventor/Applicant Name:	Jeffrey B. Etter
Customer Number:	84802
Filer:	Colin O. Hughes/Eric Baclig
Filer Authorized By:	Colin O. Hughes
Attorney Docket Number:	9516-847-999/501872-847
Receipt Date:	22-AUG-2014
Filing Date:	14-MAY-2009
Time Stamp:	17:24:00
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$960
RAM confirmation Number	4079
Deposit Account	503013
Authorized User	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)

1	Issue Fee Payment (PTO-85B)	9516-847-999_IssueFeeTrans.pdf	306222 f398dd910903c0f61d2b2e1949afa84434609391	no	1
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	30353 788a55e15f2dfcc190fc3e261cfb47feabb25bcf	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	336575		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/466,213	09/30/2014	8846628	9516-847-999/501872-847	5370

84802 7590 09/10/2014

JONES DAY for Celgene Corporation
222 E. 41ST. STREET
NEW YORK, NY 10017

ISSUE NOTIFICATION

The projected patent number and issue date are specified above.

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(application filed on or after May 29, 2000)

The Patent Term Adjustment is 260 day(s). Any patent to issue from the above-identified application will include an indication of the adjustment on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (http://pair.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Application Assistance Unit (AAU) of the Office of Data Management (ODM) at (571)-272-4200.

APPLICANT(s) (Please see PAIR WEB site http://pair.uspto.gov for additional applicants):

Jeffrey B. Etter, Boulder, CO;
Mei Lai, Longmont, CO;
Jay Thomas Backstrom, Leawood, KS;

Efile

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent of: Etter, *et al.*	Issued on: September 30, 2014
Patent No.: 8,846,628	Confirmation No.: 5370
Application No.: 12/466,213	Group Art Unit: 1673
Filed: May 14, 2009	Examiner : Lawrence E. Crane
For: ORAL FORMULATIONS OF CYTIDINE ANALOGS AND METHODS OF USE THEREOF	Attorney Docket No.: 9516-847-999 CAM: 501872-999847

REQUEST FOR CERTIFICATE OF CORRECTION UNDER 37 C.F.R. § 1.322

Attn: Certificate of Correction Branch
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Sir:

Patentees hereby respectfully request the issuance of a Certificate of Correction in connection with the above-identified patent. The correction is listed on the attached Form PTO/SB/44, which is submitted herewith. The error to be corrected is as follows:

In claim 13, at column 82, line 21, between "tablet" and "comprises", insert -- further--.

The correction above is made to conform the claim with an Examiner's amendment.

Patentees respectfully point out that the above-mentioned error is a mistake on the part of the Patent and Trademark Office. As evidence, Patentees submit herewith, as **Exhibit A**, a copy of the Examiner-Initiated Interview Summary dated May 28, 2014; and **Exhibit B**, a copy of the Notice of Allowance dated May 28, 2014.

It is shown in **Exhibit A** and on page 6 of **Exhibit B** that former claim 73 (which corresponds to patent claim 13) is to include the term "further" before the term "comprises."

Accordingly, an expedited issuance of Certificate of Correction is respectfully requested.

Patent No.: 8,846,628
Application No.: 12/466,213

Patentees believe that no fees are due. However, please charge any shortage in fees due in connection with the filing of this paper to Jones Day Deposit Account No. 50-3013, referencing our number 501872-999847 and please credit any excess fees to such deposit account.

Respectfully submitted,

Date: October 17, 2014

/Colin O. Hughes/ 72,172

By: Colin O. Hughes (Reg. No.)
For: Yeah-Sil Moon 52,042

JONES DAY
222 East 41st Street
New York, New York 10017-6702
(858) 314-1200

SDI-600220052v1
501872 - 999847

EXHIBIT A

Examiner-Initiated Interview Summary	Application No. 12/466,213	Applicant(s) ETTER ET AL.	
	Examiner Lawrence E. Crane	Art Unit 1673	

All participants (applicant, applicant's representative, PTO personnel):

(1) *Lawrence E. Crane*. (3) *Robert Chang*.

(2) *Colin Hughes*. (4)_____.

Date of Interview: *14 May 2014*.

Type: ☒ Telephonic ☐ Video Conference
☐ Personal [copy given to: ☐ applicant ☐ applicant's representative]

Exhibit shown or demonstration conducted: ☐ Yes ☒ No.
If Yes, brief description: _____.

Issues Discussed ☐101 ☒112 ☐102 ☐103 ☐Others
(For each of the checked box(es) above, please describe below the issue and detailed description of the discussion)

Claim(s) discussed: *73*.

Identification of prior art discussed: *None*.

Substance of Interview
(For each issue discussed, provide a detailed description and indicate if agreement was reached. Some topics may include: identification or clarification of a reference or a portion thereof, claim interpretation, proposed amendments, arguments of any applied references etc...)

Applicant was advised that claim 73 lacked antecedent basis but that this could be cured by adding the term "further" before the term "comprises." Applicant agreed and authorized an Examiner's Amendment.

Applicant recordation instructions: It is not necessary for applicant to provide a separate record of the substance of interview.

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

☐ Attachment

/Lawrence E. Crane/ Primary Examiner, Art Unit 1673	

EXHIBIT B

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

NOTICE OF ALLOWANCE AND FEE(S) DUE

64802 7590 05/28/2014

JONES DAY for Celgene Corporation
222 E. 41ST. STREET
NEW YORK, NY 10017

EXAMINER
CRANE, LAWRENCE E

ART UNIT	PAPER NUMBER
1673	

DATE MAILED: 05/28/2014

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/466,213	05/14/2009	Jeffrey B. Etter	9516-847-999/501872-847	5370

TITLE OF INVENTION: ORAL FORMULATIONS OF CYTIDINE ANALOGS AND METHODS OF USE THEREOF

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$960	$0	$0	$960	08/28/2014

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the ENTITY STATUS shown above. If the ENTITY STATUS is shown as SMALL or MICRO, verify whether entitlement to that entity status still applies.

If the ENTITY STATUS is the same as shown above, pay the TOTAL FEE(S) DUE shown above.

If the ENTITY STATUS is changed from that shown above, on PART B - FEE(S) TRANSMITTAL, complete section number 5 titled "Change in Entity Status (from status indicated above)".

For purposes of this notice, small entity fees are 1/2 the amount of undiscounted fees, and micro entity fees are 1/2 the amount of small entity fees.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Utility patents issuing on applications filed on or after Dec. 12, 1980 may require payment of maintenance fees. It is patentee's responsibility to ensure timely payment of maintenance fees when due.

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: Mail Mail Stop ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
or Fax (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

84892 7590 05/28/2014
JONES DAY for Celgene Corporation
222 E. 41ST. STREET
NEW YORK, NY 10017

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission
I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

(Depositor's name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/466,213	05/14/2009	Jeffrey B. Etter	9516-847-999/501872-847	5370

TITLE OF INVENTION: ORAL FORMULATIONS OF CYTIDINE ANALOGS AND METHODS OF USE THEREOF

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$960	$0	$0	$960	08/28/2014

EXAMINER	ART UNIT	CLASS-SUBCLASS
CRANE, LAWRENCE E	1673	514-043000

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).
- ☐ Change of correspondence address (or Change of Correspondence Address Form PTO/SB/122) attached.
- ☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list
(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,
(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.
1 .....
2 .....
3 .....

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE (B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent): ☐ Individual ☐ Corporation or other private group entity ☐ Government

4a. The following fee(s) are submitted:
- ☐ Issue Fee
- ☐ Publication Fee (No small entity discount permitted)
- ☐ Advance Order - # of Copies .....

4b. Payment of Fee(s): **(Please first reapply any previously paid issue fee shown above)**
- ☐ A check is enclosed.
- ☐ Payment by credit card. Form PTO-2038 is attached.
- ☐ The Director is hereby authorized to charge the required fee(s), any deficiency, or credits any overpayment, to Deposit Account Number ..... (enclose an extra copy of this form).

5. **Change in Entity Status** (from status indicated above)
- ☐ Applicant certifying micro entity status. See 37 CFR 1.29
  NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.
- ☐ Applicant asserting small entity status. See 37 CFR 1.27
  NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.
- ☐ Applicant changing to regular undiscounted fee status.
  NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature ..... Date .....

Typed or printed name ..... Registration No. .....

PTOL-85 Part B (10-13) Approved for use through 10/31/2013. OMB 0651-0033 U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/466,213	05/14/2009	Jeffrey B. Elter	9516-847-999/501872-847	5370

64802 7590 05/28/2014
JONES DAY for Celgene Corporation
222 E. 41ST. STREET
NEW YORK, NY 10017

EXAMINER	
CRANE, LAWRENCE E	

ART UNIT	PAPER NUMBER
1673	

DATE MAILED: 05/28/2014

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(Applications filed on or after May 29, 2000)

The Office has discontinued providing a Patent Term Adjustment (PTA) calculation with the Notice of Allowance.

Section 1(h)(2) of the AIA Technical Corrections Act amended 35 U.S.C. 154(b)(3)(B)(i) to eliminate the requirement that the Office provide a patent term adjustment determination with the notice of allowance. See Revisions to Patent Term Adjustment, 78 Fed. Reg. 19416, 19417 (Apr. 1, 2013). Therefore, the Office is no longer providing an initial patent term adjustment determination with the notice of allowance. The Office will continue to provide a patent term adjustment determination with the Issue Notification Letter that is mailed to applicant approximately three weeks prior to the issue date of the patent, and will include the patent term adjustment on the patent. Any request for reconsideration of the patent term adjustment determination (or reinstatement of patent term adjustment) should follow the process outlined in 37 CFR 1.705.

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

OMB Clearance and PRA Burden Statement for PTOL-85 Part B

The Paperwork Reduction Act (PRA) of 1995 requires Federal agencies to obtain Office of Management and Budget approval before requesting most types of information from the public. When OMB approves an agency request to collect information from the public, OMB (i) provides a valid OMB Control Number and expiration date for the agency to display on the instrument that will be used to collect the information and (ii) requires the agency to inform the public about the OMB Control Number's legal significance in accordance with 5 CFR 1320.5(b).

The information collected by PTOL-85 Part B is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450. Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Notice of Allowability	Application No. 12/466,213	Applicant(s) ETTER ET AL.	
	Examiner Lawrence E. Crane	Art Unit 1673	AIA (First Inventor to File) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to *the amendment filed February 27, 2014*.
   ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on_____.
2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.
3. ☒ The allowed claim(s) is/are *1,6-11,13-23,33-37,41-50 and 73-82*. As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.
4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
   Certified copies:
   a) ☐ All b) ☐ Some * c) ☐ None of the:
      1. ☐ Certified copies of the priority documents have been received.
      2. ☐ Certified copies of the priority documents have been received in Application No. _____.
      3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).
   * Certified copies not received: _____.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____.
   Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).
6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)
2. ☒ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date 20140227
3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material
4. ☒ Interview Summary (PTO-413), Paper No./Mail Date *20140514*.
5. ☒ Examiner's Amendment/Comment
6. ☒ Examiner's Statement of Reasons for Allowance
7. ☐ Other _____.

/Lawrence E. Crane/
Primary Examiner, Art Unit 1673

The present application is being examined under the pre-AIA first to invent provisions.

The Group and/or Art Unit location of your application in the PTO has changed. To aid in correlating any papers for this application, all further correspondence regarding this application should be directed to Group 1600, Art Unit **1673**.

An Examiner's Amendment to the record appears below. Should the changes and/or additions be unacceptable to applicant, an amendment may be filed as provided by 37 C.F.R. §1.312. To ensure consideration of such an amendment, it **MUST** be submitted no later than the payment of the Issue Fee.

In claim **73** at line 1, the term "tablet comprises" was amended to read -- tablet further comprises --.

Authorization for this Examiner's Amendment was given in a telephone interview with Colin Hughes and confirmed by Robert Chang on May 14, 2014.

The following is an Examiner's Statement of Reasons for Allowance:

Examiner has reviewed the previous Office action, the above Examiner's Amendment (necessary to address an issue of inadequate antecedent basis), applicant's response including an extensive and detailed declaration filed under 37 C.F.R. § 1.132 filed February 27, 2014, and an additional or supplemental Information Disclosure Statement filed February 27, 2014 citing references relied upon extensively by the noted declaration. The declarant, Mssr. C. L. Beach, has argued in detail in opposition to examiner's rejections citing prior art, noting that the construction of the rejections of record are inappropriate in view of the prior art cited, and that this conclusion is buttressed in several different ways by the detailed analysis of the instant cited prior art presented in the declaration. Additionally data presented in the declaration, and also in the application, noting the surprisingly high anti-neoplastic medicinal activities of the claimed non-enteric coated pharmaceutical composition, has been identified as unexpected, and therefore said data is also a proper basis to argue in favor of a conclusions opposite to the previously asserted conclusions of obviousness.

Additionally examiner notes that applicant's amendments to the claims together with applicant's arguments made in parallel with the noted declaration have rendered all of the

rejections citing prior art, rejections citing 35 U.S.C. §112, and all objections of record moot. Therefore, all rejections and objections of record have been withdrawn. There remaining no recognized bases for objection to, or rejection of, the claims remaining of record, said claims have been found allowable as presently amended.

Any comments considered necessary by applicant must be submitted no later than the payment of the Issue Fee and, to avoid processing delays, should preferably **accompany** the Issue Fee. Such submissions should be clearly labeled "Comments on Statement of Reasons for Allowance."

Papers related to this application may be submitted to Group 1600 via facsimile transmission(FAX). The transmission of such papers must conform with the notice published in the Official Gazette (1096 OG 30, November 15, 1989). The telephone number for sending a FAX (unofficially) directly to Examiner's computer in Group 1600 is **571-273-0651**. The telephone number for sending an Official FAX to the PTO is 571-273-8300.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Examiner L. E. Crane whose telephone number is **571-272-0651**. The examiner can normally be reached between 9:30 AM and 5:00 PM, Monday through Friday.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Ms. S. Anna Jiang, can be reached on **571-272-0627**.

Any inquiry of a general nature or relating to the status of this application should be directed to the Group 1600 receptionist whose telephone number is **571-272-1600**.

All Post-Allowance Correspondence concerning this application must be mailed to:

BOX ISSUE FEE
COMMISSIONER FOR PATENTS
WASHINGTON, DC 20231

OR you can FAX them to the Office of Patent Publications at 571-273-8300, in order to expedite the handling of such correspondence as amendments under 37 C.F.R. §1.312;

Information Disclosure Statements (IDS's), and formal drawings. Sending Post-Allowance papers to Technology Center 1600 will only cause delays in matching papers with the case.

For information concerning status of correspondence sent after receipt of the Notice of Allowance, please contact the Correspondence Branch at **571-272-4200**. The Notice of Allowance also has an insert containing contact information of other items, including Issue Fees, receipt of formal drawings, and the status of the application.

LECrane:lec
05/15/2013

/Lawrence E. Crane/

Primary Examiner, Art Unit 1673

L. E. Crane
Primary Patent Examiner
Technology Center 1600

PTO/SB/44 (09-07)
Approved for use through 08/31/2013. OMB 0651-0033
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

(Also Form PTO-1050)

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 8,846,628 B2

APPLICATION NO.: 12/466,213

ISSUE DATE : September 30, 2014

INVENTOR(S) : Jeffrey B. ETTER; Mei LAI; Jay Thomas BACKSTROM

It is certified that an error appears or errors appear in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In claim 13, at column 82, line 21, between "tablet" and "comprises", insert -- further --

MAILING ADDRESS OF SENDER (Please do not use customer number below):

Jones Day
222 E. 41st Street
New York, NY 10017-6702

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Acknowledgement Receipt

EFS ID:	20453128
Application Number:	12466213
International Application Number:	
Confirmation Number:	5370
Title of Invention:	ORAL FORMULATIONS OF CYTIDINE ANALOGS AND METHODS OF USE THEREOF
First Named Inventor/Applicant Name:	Jeffrey B. Etter
Customer Number:	84802
Filer:	Colin O. Hughes/Keiko Masuyama Hicks
Filer Authorized By:	Colin O. Hughes
Attorney Docket Number:	9516-847-999/501872-847
Receipt Date:	17-OCT-2014
Filing Date:	14-MAY-2009
Time Stamp:	20:11:20
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Request for Certificate of Correction	9516-847-999_Request_Certificate_Correction.pdf	2094712 85b03f9f9dc5e00f01118bfff8380cc5e025453a	no	13

Warnings:

Information:

2	Request for Certificate of Correction	9516-847-999_SB44.pdf	155349 a6aa71a8d8b51d43b7e264f1b5637d33883825e6	no	2
Warnings:					
Information:					
			Total Files Size (in bytes):	2250061	

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO.	: 8,846,628 B2
APPLICATION NO.	: 12/466213
DATED	: September 30, 2014
INVENTOR(S)	: Jeffrey B. Etter et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In the Claims

In claim 13, at column 82, line 21, between "tablet" and "comprises", insert -- further --

Signed and Sealed this
Thirtieth Day of December, 2014

Michelle K. Lee

Michelle K. Lee
Deputy Director of the United States Patent and Trademark Office

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	ETTER et al.	Confirmation No.:	5370
Patent No.	8,846,628	Art Unit:	1673
Issued:	September 30, 2014	Examiner:	CRANE, Lawrence E.
Serial No.:	12/466,213		
Filed:	May 14, 2009		
For:	ORAL FORMULATIONS OF CYTIDINE ANALOGS AND METHODS OF USE THEREOF	Attorney Docket No.: (CAM:	9516-847-999 501872-999847)

REQUEST FOR RECONSIDERATION OF PATENT TERM ADJUSTMENT UNDER 37 C.F.R. § 1.705(b)

Filed via EFS-Web
Mail Stop PETITION
Commissioner for Patents
PO BOX 1450
Alexandria, Virginia 22313-1450

Sir:

The above-identified patent application, U.S. Patent Application No. 12/466,213 (hereinafter "the '213 application"), issued as U.S. Patent No. 8,846,628 (hereinafter "the '628 patent") on September 30, 2014. Patentee hereby respectfully requests reconsideration of the patent term adjustment (PTA) indicated on the '628 patent. The '628 patent indicates patent term adjustment under 35 U.S.C. § 154(b) of 260 days. However, Patentee believes that the patent term adjustment should be **385** days.

A request for reconsideration of patent term adjustment under 37 C.F.R. § 1.705(b) must be filed within two months of the date the patent issued (extension available) and must comply with the requirements of 37 C.F.R. § 1.705(b), which are detailed below:

(1) the fee set forth in 37 C.F.R. § 1.18(e); and

(2) a statement of the facts involved, specifying:

(i) the correct patent term adjustment and the basis or bases under 37 C.F.R. § 1.702 for the adjustment;

(ii) the relevant dates as specified in 37 C.F.R. § 1.703(a) through (e) for which an adjustment is sought and the adjustment as specified in 37 C.F.R. § 1.703(f) to which the patent is entitled;

(iii) whether the patent is subject to a terminal disclaimer and any expiration date specified in the terminal disclaimer; and

(iv)(A) any circumstances during the prosecution of the application resulting in the patent that constitute a failure to engage in reasonable efforts to conclude processing or examination of such application as set forth in 37 C.F.R. § 1.704; or

(B) that there were no circumstances constituting a failure to engage in reasonable efforts to conclude processing or examination of such application as set forth in 37 C.F.R. § 1.704.

See 37 C.F.R. § 1.705(b).

The present request for reconsideration of PTA is accompanied by a request for extension of time of four (4) months, which will extend the request period from November 30, 2014, *i.e.*, the date that is two months from the date the patent issued, to and including March 30, 2015, with authorization to charge the required extension of time fee. Thus, the present request for reconsideration of PTA is timely under 37 C.F.R. § 1.705(b).

In support of this request, Patentee submits the following:

1. Fee Required Under 37 C.F.R. § 1.705(b)(1)

Pursuant to 37 C.F.R. §§ 1.705(b)(1) and 1.18(e), it is estimated that the fee for filing this request is **$2,400**. The Director is hereby authorized to charge the required fee(s) to Jones Day Deposit Account No. 50-3013 (referencing 501872-999847). In the event any additional fees are required, please charge them to Jones Day Deposit Account No. 50-3013.

2. Statement Required Under 37 C.F.R. § 1.705(b)(2)

Pursuant to 37 C.F.R. § 1.705(b)(2), Patentee submits the following statement of facts in support of this request:

(i) The correct patent term adjustment is **385** days, and the bases under 37 C.F.R. § 1.702 for this patent term adjustment are attributable to:

(a) "**A period delay**": the failure of the USPTO to mail a notification under 35 U.S.C. § 132 not later than fourteen months after the date on which the '213 application was filed, and the failure of the USPTO to respond to a reply under 35 U.S.C. § 132 not later than four months after the date on which the reply was filed (delay of 652 days under 37 C.F.R. § 1.702(a));

(b) "**B period delay**": the failure of the USPTO to issue a patent within three years after the date on which the application was filed (delay of 205 days, under 37 C.F.R. § 1.702(b));

(c) reduction of 0 days of overlap between the A period delay and the B period delay, and total non-overlapping A and B period delays are 857 days (652 days of A period delay plus 205 days of B period delay);

(d) reduction of period of adjustment of patent term under 37 C.F.R. § 1.704 of 472 days; and

(e) the correct patent term adjustment is 385 days (857 days minus 472 days).

(ii) The relevant dates as specified in 37 C.F.R. §§ 1.703(a) through (e) for which an adjustment is sought and the adjustment as specified in 37 C.F.R. § 1.703(f) are as follows:

A. Adjustment under 37 C.F.R. §§ 1.703(a) through (e):

1. 37 C.F.R. § 1.703(a):

37 C.F.R. § 1.703(a) provides, in relevant part, that the period of adjustment is the sum of:

> (1) The number of days, if any, in the period beginning on the day after the date that is fourteen months after the date on which the application was filed under 35 U.S.C. 111(a) or the date the national stage commenced under 35 U.S.C. 371(b) or (f) in an international application and ending on the date of mailing of either an action under 35 U.S.C. 132, or a notice of allowance under 35 U.S.C. 151, whichever occurs first;
>
> (2) The number of days, if any, in the period beginning on the day after the date that is four months after the date a reply under § 1.111 was filed and ending on the date of mailing of either an action under 35 U.S.C. 132, or a notice of allowance under 35 U.S.C. 151, whichever occurs first;
>
> ....

The patent term adjustment under 37 C.F.R. § 1.703(a) includes the period beginning July 15, 2010 (the day after fourteen months after the '213 application was filed), and ending August 1, 2011 (the date a non-final rejection was mailed from the USPTO). This period consists of 383 days.

The patent term adjustment under 37 C.F.R. § 1.703(a) further includes the period beginning December 4, 2012 (the day after four months after the date on which a reply was filed), and ending August 29, 2013 (the date a non-final rejection was mailed from the USPTO). This period consists of 269 days. Therefore, A period of delay is a total (383+269) of 652 days, as correctly determined on the USPTO's "Patent Term Adjustments" page (a copy of which is enclosed herewith as Appendix A).

Therefore, the total A period delay under 37 C.F.R. § 1.703(a) is 652 days.

2. 37 C.F.R. § 1.703(b):

37 C.F.R. § 1.703(b) provides, in relevant part, that

> The period of adjustment under § 1.702(b) is the number of days, if any, in the period beginning on the day after the date that is three years after the date on which the application was filed under 35 U.S.C. 111(a) or the national stage commenced under 35 U.S.C. 371(b) or (f) in an international application and ending on the date a patent was issued, but not including the sum of the following periods:
>
> (1) The number of days, if any, in the period beginning on the date on which a request for continued examination of the application under 35 U.S.C. 132(b) was filed and ending on the date the patent was issued;
>
> ….

The USPTO determined that the patent term adjustment under 37 C.F.R. § 1.703(b) is 80 days, corresponding to the period from May 15, 2012 (the day after the date that is 3 years after the date on which the '213 application was filed (*i.e.*, May 14, 2009)) to August 2, 2012 (the day before the date on which a request for continued examination ("RCE") was filed). *See* USPTO's PAIR's "Patent Term Adjustments" page (a copy of which is enclosed herewith as Appendix A). However, Patentee asserts that the correct patent term adjustment under 37 C.F.R. § 1.703(b) is 205 days, as discussed in more detail below.

The '213 application was filed on May 14, 2009, and the '628 patent issued on September 30, 2014, which is after the date that is three years after the date on which the '213 application was filed. Therefore, patent term adjustment is provided for every day beginning on May 15, 2012 (the day after the date that is 3 years from filing of the application) until September 30, 2014 (the date of issuance of the patent), but not including the period beginning on August 3, 2012 (the date RCE was filed) and ending on May 28, 2014 (the date the first Notice of Allowance was mailed). *See Novartis AG v. Lee*, 740 F.3d 593 (Fed. Cir. 2014).

The USPTO's and Patentee's calculations of the patent term adjustment under 37 C.F.R. § 1.703(b) diverge due to differences in interpretation of the phrase "any time consumed by continued examination" in 35 U.S.C. § 154(b)(1)(B). Patentee submits that

the USPTO's determination of patent term adjustment under 37 C.F.R. § 1.703(b)(1) is inconsistent with the requirements of 35 U.S.C. § 154(b)(1)(B).

35 U.S.C. § 154(b)(1)(B) specifies, in relevant part, that

> Subject to the limitations under paragraph (2), if the issue of an original patent is delayed due to the failure of the United States Patent and Trademark Office to issue a patent within 3 years after the actual filing date of the application under section 111(a) in the United States … not including—
>
> (i) any time consumed by continued examination of the application requested by the applicant under section 132(b);
> …
> the term of the patent shall be extended 1 day for each day after the end of that 3-year period until the patent is issued.

The USPTO's interpretation of 35 U.S.C. § 154(b)(1)(B) categorically treats all days after the RCE filing until patent issuance as "time consumed by continued examination," *including the time between mailing of a Notice of Allowance and patent's issuance*. *See* 37 C.F.R. § 1.703(b)(1). Consistent with this interpretation, the USPTO's determination of 80 days of patent term adjustment under 37 C.F.R. § 1.703(b) in the '628 patent is based on inclusion of the time between mailing of the Notice of Allowance and the patent's issuance into the "time consumed by continued examination," which was subtracted by the USPTO from the B period delay.

By contrast, Patentee submits that, the period consumed by continued examination in determining the B period delay is limited to the number of days in the period beginning on the date on which the RCE was filed and ending on the date of the mailing of the Notice of Allowance, which is an action that closes prosecution, not on the issue date as set forth by the USPTO.

In *Novartis*, the Federal Circuit agreed with the patentee/appellant that time consumed by continued examination under 35 U.S.C. § 154(b)(1)(B) concludes once a notice of allowance is issued. *Novartis*, 740 F.3d at 602. In particular, the Court stated:

> "We reject the PTO's view that the time after allowance, until issuance, is 'time consumed by continued examination' and so is excluded from adjustments given to the patentee. Such time from allowance to issuance undisputedly would count toward the PTO's three-year allotment in a case not involving a continued examination. There is no basis for distinguishing a continued examination case."
>
> "An 'examination' presumptively ends at allowance, when prosecution is closed and there is no further examination on the merits in the absence of a special reopening."
>
> "The commonsense understanding of 'time consumed by continued examination,' 35 U.S.C. § 154(b)(1)(B)(i), is time up to allowance, but not later, unless examination on the merits resumes." *Id.*

Consistent with this interpretation, the first Notice of Allowance mailed on May 28, 2014 for the '213 application expressly stated that "THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED." (First underline added).

Under *Novartis*, and now under 37 C.F.R. §§ 1.703 and 1.704 (see Federal Register, Vol. 80, No. 6, page 1346 of January 9, 2015) the USPTO's inclusion of the period from the mailing date of the Notice of Allowance to the issue date as part of the period consumed by continued examination is inconsistent with the requirements of 35 U.S.C. § 154(b)(1)(B). Thus, Patentee submits that the time period between the mailing of the Notice of Allowance (*i.e.,* May 28, 2014) to the issue date (*i.e.,* September 30, 2014), totaling 126 days, must be counted as part of the B period delay since the patent issuance was further delayed and still did not issue within the 3-year period guaranteed by the statute. 35 U.S.C. § 154(b)(1)(B).

In this case, the time in the period beginning with the day after the date that is three years after the '213 application was filed (*i.e.*, May 15, 2012) and ending with the issuance of the patent on September 30, 2014 is 869 days. The time consumed by continued examination is the period beginning with the RCE filing on August 3, 2012

and ending with the mailing of the Notice of Allowance on May 28, 2014, totaling 664 days. Therefore, the correct patent term adjustment for the B period delay under § 1.703(b) is 205 days (869 days minus 664 days).

In view of the foregoing, Patentee submits that the correct patent term adjustment for the B period delay is 205 days.

3. 37 C.F.R. §§ 1.703(c)-(e): There is no patent term adjustment under these subsections.

B. Adjustment under 37 C.F.R. § 1.703(f):

According to 37 C.F.R. § 1.703(f): "The term of a patent entitled to adjustment under § 1.702 and this section shall be adjusted for the sum of the periods calculated under paragraphs (a) through (e) of this section, to the extent that such periods are not overlapping, less the sum of the periods calculated under § 1.704." *See* 37 C.F.R. § 1.703(f).

There are no overlapping days between the delay under 37 C.F.R. § 1.703(a) and the delay under 37 C.F.R. § 1.703(b), because the calendar days for the A period delay fall within the period not included in the B period delay.

The total USPTO delay under 37 C.F.R. §§ 1.703(a)-(e) for the adjustment should be a summation of the delays under 37 C.F.R. § 1.703(a) and 37 C.F.R. § 1.703(b) minus the overlapping days between the period of such delays, or 857 days (the sum of 652 days A period delays and 205 days B period delays, minus 0 overlap days).

As discussed in section (iv) below, the total delay attributable to Applicant under 37 C.F.R. § 1.704 is calculated to amount to 472 days.

Accordingly, Patentee believes that the total period of adjustment due under 37 C.F.R. § 1.703(f) is **385** days, which is the sum of the delays under 37 C.F.R. §§ 1.703(a) through (e) (*i.e.*, 857 days) less the sum of the periods calculated under Section 1.704 (*i.e.*, 472 days).

(iii) Terminal Disclaimer:

The '628 patent is not subject to a terminal disclaimer.

(iv) Circumstances that Constitute a Failure to Engage in Reasonable Efforts to conclude Processing or Examination of the Application as Set Forth in 37 C.F.R. § 1.704:

The circumstances that relate to failure of Applicant to engage in reasonable efforts to conclude processing or examination of the present application as set forth in 37 C.F.R. § 1.704 are described below:

(a). 30 day delay to respond to the non-final Office Action mailed on August 1, 2011. While the three month period from the mailing date of the non-final Office Action expired on November 1, 2011, Applicant filed the response on December 1, 2011.

(b). 66 day delay to respond to the final Office Action mailed on February 28, 2012. While the three month period from the mailing date of the final Office Action expired on May 28, 2012, Applicant filed the response on August 3, 2012.

(c). 286 day delay to file an Information Disclosure Statement ("IDS") after the filing of the response on August 3, 2012, as determined by the USPTO. Applicant filed the IDS on May 16, 2013.

(d). 90 day delay to respond to the non-final Office Action mailed on August 29, 2013, as determined by the USPTO. While the three month period from the mailing date of the non-final Office Action expired on November 29, 2013, Applicant filed the response on February 27, 2014.

Accordingly, the delay attributed to Applicant under 37 C.F.R. § 1.704 totals 472 days (the sum of 30 days plus 66 days plus 286 days plus 90 days), as correctly determined by the USPTO PAIR webpage for the '213 application.

3. Correct Days of Patent Term Adjustment

In summary, Patentee submits that:

(i) the total A period delay by the USPTO under 37 C.F.R. § 1.702(a) is 652 days;

(ii) the total B period delay by the USPTO under 37 C.F.R. § 1.702(b) is 205 days;

(iii) the overlapping days between the A period delay and the B period delay is 0 days.

(iv) the total delay attributable to Applicant under 37 C.F.R. § 1.704 is 472 days; and

(v) the total period of adjustment due under 37 C.F.R. § 1.703(f) is 385 days (sum of 652 days and 205 days, minus 472 days).

Accordingly, Patentee respectfully requests an adjustment of patent term totaling **385 days**.

Patentee respectfully requests approval and entry of this request. The USPTO is invited to contact the undersigned regarding any questions concerning any of the above.

Respectfully submitted,

Date: March 27, 2015

/Colin O. Hughes/ 72,172

By: Colin O. Hughes (Reg. No.)

For Yeah-Sil Moon 52,042

JONES DAY
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Appendix A

12/466,213	ORAL FORMULATIONS OF CYTIDINE ANALOGS AND METHODS OF USE THEREOF	9516-847-999/501672-847 03-26-2015::19:25:11

Patent Term Adjustments

Patent Term Adjustment (PTA) for Application Number: 12/466,213

Filing or 371(c) Date:	05-14-2009	Overlapping Days Between {A and B} or {A and C}:	0
Issue Date of Patent:	09-30-2014	Non-Overlapping USPTO Delays:	732
A Delays:	652	PTO Manual Adjustments:	0
B Delays:	80	Applicant Delays:	472
C Delays:	0	Total PTA Adjustments:	260

Patent Term Adjustment History Explanation Of Calculations

Number	Date	Contents Description	PTO (Days)	APPL (Days)	Start
87.5	08-02-2012	PTA 36 Months	80		0.5
87	09-30-2014	Patent Issue Date Used in PTA Calculation			0
86	08-27-2014	Export to Final Data Capture			0
85	08-26-2014	Dispatch to FDC			0
84	08-25-2014	Application Is Considered Ready for Issue			0
83	08-22-2014	Issue Fee Payment Verified			0
82	08-22-2014	Issue Fee Payment Received			0
81	07-16-2014	Finished Initial Data Capture			0
80	05-28-2014	Export to Initial Data Capture			0
79	05-28-2014	Mail Notice of Allowance			0
78	05-27-2014	Office Action Review			0
77	05-27-2014	Office Action Review			0
76	05-27-2014	Office Action Review			0
75	05-27-2014	Office Action Review			0
74	05-27-2014	Office Action Review			0
73	05-27-2014	Issue Revision Completed			0
72	05-27-2014	Document Verification			0
71	05-27-2014	Notice of Allowance Data Verification Completed			0

70	05-20-2014	Interview Summary - Examiner Initiated - Telephonic			0
69	05-20-2014	Interview Summary - Examiner Initiated			0
68	05-20-2014	Reasons for Allowance			0
67	05-20-2014	Examiner's Amendment Communication			0
66	05-20-2014	Allowability Notice			0
65	05-12-2014	Information Disclosure Statement considered			0
64	02-27-2014	Affidavit(s) (Rule 131 or 132) or Exhibit(s) Received			0
63	02-28-2014	Date Forwarded to Examiner			0
62	02-27-2014	Response after Non-Final Action		90	51
61	02-27-2014	Request for Extension of Time - Granted			0
60	02-27-2014	Electronic Information Disclosure Statement			0
59	02-27-2014	Information Disclosure Statement (IDS) Filed			0
58	02-03-2014	Mail Interview Summary - Applicant Initiated - Personal			0
57	01-30-2014	Office Action Review			0
56	01-30-2014	Office Action Review			0
55	01-30-2014	Office Action Review			0
54	01-29-2014	Interview Summary - Applicant Initiated - Telephonic			0
53	01-29-2014	Interview Summary- Applicant Initiated			0
52	01-29-2014	Interview Summary - Applicant Initiated - Personal			0
51	08-29-2013	Mail Non-Final Rejection	269		38
50	08-26-2013	Office Action Review			0
49	08-26-2013	Non-Final Rejection			0
48	05-16-2013	Information Disclosure Statement considered			0
47	09-05-2012	Information Disclosure Statement considered			0
46	05-16-2013	Reference capture on IDS			0

45	05-16-2013	Information Disclosure Statement (IDS) Filed		286	38
44	05-16-2013	Information Disclosure Statement (IDS) Filed			0
43	09-05-2012	Reference capture on IDS			0
42	09-05-2012	Information Disclosure Statement (IDS) Filed			0
41	09-05-2012	Information Disclosure Statement (IDS) Filed			0
40	08-06-2012	Date Forwarded to Examiner			0
39	08-03-2012	Amendment Submitted/Entered with Filing of CPA/RCE			0
38	08-03-2012	Request for Continued Examination (RCE)		66	34
37	08-06-2012	Disposal for a RCE / CPA / R129			0
36	08-03-2012	Request for Extension of Time - Granted			0
35	08-03-2012	Workflow - Request for RCE - Begin			0
34	02-28-2012	Mail Final Rejection (PTOL - 326)			0
33	02-27-2012	Office Action Review			0
32	02-24-2012	Final Rejection			0
31	12-01-2011	Information Disclosure Statement considered			0
30	12-01-2011	Reference capture on IDS			0
29	12-02-2011	Date Forwarded to Examiner			0
28	12-01-2011	Response after Non-Final Action		30	25
27	12-01-2011	Request for Extension of Time - Granted			0
26	12-01-2011	Information Disclosure Statement (IDS) Filed			0
25	08-01-2011	Mail Non-Final Rejection	383		0.5
24	07-29-2011	Office Action Review			0
23	07-25-2011	Non-Final Rejection			0
22	04-02-2010	Information Disclosure Statement considered			0
18	01-14-	Case Docketed to Examiner in GAU			0

	2011		
17	04-02-2010	Reference capture on IDS	0
16	04-02-2010	Information Disclosure Statement (IDS) Filed	0
15	04-02-2010	Information Disclosure Statement (IDS) Filed	0
14	11-19-2009	PG-Pub Issue Notification	0
13	08-12-2009	Change in Power of Attorney (May Include Associate POA)	0
12	08-10-2009	Correspondence Address Change	0
11	08-04-2009	Application Dispatched from OIPE	0
10	07-28-2009	Sent to Classification Contractor	0
9	07-28-2009	Filing Receipt - Updated	0
8	07-21-2009	Additional Application Filing Fees	0
7	07-21-2009	A statement by one or more inventors satisfying the requirement under 35 USC 115, Oath of the Applic	0
6	07-21-2009	Applicant has submitted new drawings to correct Corrected Papers problems	0
5	05-28-2009	Filing Receipt	0
4	05-28-2009	Notice Mailed--Application Incomplete--Filing Date Assigned	0
3	05-15-2009	Cleared by OIPE CSR	0
2	05-15-2009	IFW Scan & PACR Auto Security Review	0
1	05-14-2009	Initial Exam Team nn	0
0.5	05-14-2009	Filing date	0

Close Window

Electronic Patent Application Fee Transmittal

Application Number:	12466213
Filing Date:	14-May-2009
Title of Invention:	ORAL FORMULATIONS OF CYTIDINE ANALOGS AND METHODS OF USE THEREOF
First Named Inventor/Applicant Name:	Jeffrey B. Etter
Filer:	Colin O. Hughes/Christina Quatrino
Attorney Docket Number:	9516-847-999/501872-847

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Application for patent term adjustment	1455	1	200	200
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Extension - 4 months with $0 paid	1254	1	2200	2200
Miscellaneous:				
		Total in USD ($)		**2400**

Electronic Acknowledgement Receipt

EFS ID:	21903999
Application Number:	12466213
International Application Number:	
Confirmation Number:	5370
Title of Invention:	ORAL FORMULATIONS OF CYTIDINE ANALOGS AND METHODS OF USE THEREOF
First Named Inventor/Applicant Name:	Jeffrey B. Etter
Customer Number:	84802
Filer:	Colin O. Hughes/Gilbert Martinez
Filer Authorized By:	Colin O. Hughes
Attorney Docket Number:	9516-847-999/501872-847
Receipt Date:	27-MAR-2015
Filing Date:	14-MAY-2009
Time Stamp:	18:41:39
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$2400
RAM confirmation Number	5104
Deposit Account	503013
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: Charge any Additional Fees required under 37 C.F.R. Section 1.16 (National application filing, search, and examination fees) Charge any Additional Fees required under 37 C.F.R. Section 1.17 (Patent application and reexamination processing fees)	

Charge any Additional Fees required under 37 C.F.R. Section 1.19 (Document supply fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.20 (Post Issuance fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.21 (Miscellaneous fees and charges)

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Extension of Time	9516-847-999_Petition_EOT.pdf	187243 6e46149bf347259986928a68187d8d03568 5d1fe	no	2
Warnings:					
Information:					
2	Patent Term Adjustment Petition	9516-847-999_Request.pdf	665191 da9ec34564bbb3e6f9580802d385f8264bd 78cac	no	14
Warnings:					
Information:					
3	Fee Worksheet (SB06)	fee-info.pdf	32782 e22597290172ab3742dd884931d99842eb e04516	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	885216		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/SB/22 (03-13)
Approved for use through 3/31/2013. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a)

Docket Number (Optional): 9516-847-999

Application Number: 12/466,213

Filed: May 14, 2009

For: ORAL FORMULATIONS OF CYTIDINE ANALOGS AND METHODS OF USE THEREOF

Art Unit: 1673

Examiner: Lawrence E. CRANE

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above-identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

	Fee	Small Entity Fee	Micro Entity Fee	
☐ One month (37 CFR 1.17(a)(1))	$200	$100	$50	$
☐ Two months (37 CFR 1.17(a)(2))	$600	$300	$150	$
☐ Three months (37 CFR 1.17(a)(3))	$1,400	$700	$350	$
☑ Four months (37 CFR 1.17(a)(4))	$2,200	$1,100	$550	$ 2200
☐ Five months (37 CFR 1.17(a)(5))	$3,000	$1,500	$750	$

☐ Applicant asserts small entity status. See 37 CFR 1.27.

☐ Applicant certifies micro entity status. See 37 CFR 1.29.
Form PTO/SB/15A or B or equivalent must either be enclosed or have been submitted previously.

☐ A check in the amount of the fee is enclosed.

☐ Payment by credit card. Form PTO-2038 is attached.

☐ The Director has already been authorized to charge fees in this application to a Deposit Account.

☑ The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 503013.

☑ Payment made via EFS-Web.

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

I am the

☐ applicant/inventor.

☐ assignee of record of the entire interest. See 37 CFR 3.71. 37 CFR 3.73(b) statement is enclosed (Form PTO/SB/96).

☑ attorney or agent of record. Registration number 72,172.

☐ attorney or agent acting under 37 CFR 1.34. Registration number _____.

/Colin O. Hughes/ — Signature

March 27, 2015 — Date

Colin O. Hughes (for Yeah-Sil Moon, Reg. No. 52,042) — Typed or printed name

858-314-1200 — Telephone Number

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. Submit multiple forms if more than one signature is required, see below*.

☐ * Total of _____ forms are submitted.

Privacy Act Statement

The **Privacy Act of 1974** (**P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/466,213	05/14/2009	Jeffrey B. Etter	9516-847-999/501872-847	5370

84802 7590 06/23/2015
JONES DAY for Celgene Corporation
222 E. 41ST. STREET
NEW YORK, NY 10017

EXAMINER	
CRANE, LAWRENCE E	

ART UNIT	PAPER NUMBER
1673	

MAIL DATE	DELIVERY MODE
06/23/2015	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner for Patents
United States Patent and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450
www.uspto.gov

In re Patent No. 8,846,628
Issue Date: September 30, 2014
Application No. 12/466,213
Filed: May 14, 2009
Attorney Docket No. 9516-847-999
(CAM: 501872-999847)

REDETERMINATION
OF PATENT TERM ADJUSTMENT
AND
NOTICE OF INTENT TO ISSUE
CERTIFICATE OF CORRECTION

This is a response to the "REQUEST FOR RECONSIDERATION OF PATENT TERM ADJUSTMENT UNDER 37 C.F.R. § 1.705(b)" filed March 27, 2015 requesting that the Office adjust the PTA from 260 days to 385 days. The Office has re-determined the PTA to be 385 days.

The petition is **GRANTED**.

This redetermination of patent term adjustment is not the Director's decision on the applicant's request for reconsideration within the meaning of 35 U.S.C. 154(b)(4) that triggers a 180-day period for applicant disagreeing with the Office redetermination to commence a civil action in the District Court for the Eastern District of Virginia.

The Office acknowledges submission of the $200.00 fee set forth in 37 CFR 1.18(e). The fee set forth in 37 CFR 1.18(e) is a requirement and will not be refunded. Additionally, the submission of the four month extension of time fees is acknowledged. No additional fees are required.

Relevant Procedural History

On September 30, 2014, this patent issued with a patent term adjustment determination of 260 days. On March 27, 2015, with a four month extension of time, patentee filed a "REQUEST FOR RECONSIDERATION OF PATENT TERM ADJUSTMENT UNDER 37 C.F.R. § 1.705(b)" seeking an adjustment of the determination to 385 days.

Patentee argues "The USPTO determined that the patent term adjustment under 37 C.F.R. § 1.703(b) is 80 days, corresponding to the period from May 15, 2012 (the day after the date that is 3 years after the date on which the '213 application was filed (i.e., May 14, 2009)) to August 2, 2012 (the day before the date on which a request for continued examination ("RCE") was filed). See USPTO's PAIR's "Patent Term Adjustments" page (a copy of which is enclosed herewith as Appendix A). However, Patentee asserts that the correct patent term adjustment under 37 C.F.R. § 1.703(b) is 205 days, as discussed in more detail below. The '213 application was filed on May 14, 2009, and the '628 patent issued on September 30, 2014, which is after the date that is three years after the date on which the '213 application was filed. Therefore, patent term

adjustment is provided for every day beginning on May 15, 2012 (the day after the date that is 3 years from filing of the application) until September 30, 2014 (the date of issuance of the patent), but not including the period beginning on August 3, 2012 (the date RCE was filed) and ending on May 28, 2014 (the date the first Notice of Allowance was mailed). See Novartis AG v. Lee, 740 F.3d 593 (Fed. Cir. 2014). The USPTO's and Patentee's calculations of the patent term adjustment under 37 C.F.R. § 1.703(b) diverge due to differences in interpretation of the phrase "any time consumed by continued examination" in 35 U.S.C. § 154(b)(1)(B). In this case, the time in the period beginning with the day after the date that is three years after the '213 application was filed (i.e., May 15, 2012) and ending with the issuance of the patent on September 30, 2014 is 869 days. The time consumed by continued examination is the period beginning with the RCE filing on August 3, 2012 and ending with the mailing of the Notice of Allowance on May 28, 2014, totaling 664 days. Therefore, the correct patent term adjustment for the B period delay under § 1.703(b) is 205 days (869 days minus 664 days). In view of the foregoing, Patentee submits that the correct patent term adjustment for the B period delay is 205 days."

Upon review, the USPTO finds that patentee is entitled to 385 days of PTA. The Office has revisited the amount of "B" delay under 35 U.S.C. § 154(b)(1)(B) and the amount of overlapping days under 35 U.S.C. § 154(b)(2)(A) pursuant to the Federal Circuit's decision in *Novartis AG v. Lee*, 740 F.3d 593 (Fed. Cir. 2014).

The Office notes that the interpretation of the "B" delay was based upon rule 37 CFR 1.703(b)(1) which excluded from the amount of "B" delay the period beginning on the date of filing of the continued examination and ending on the date of the issuance of the patent. However, subsequent to the filing of this lawsuit and remand to the Office, the Federal Circuit reviewed the statutory interpretation of 35 U.S.C. § 154(b)(1)(B)(i) and issued a decision regarding the effects of a Request for Continued Examination ("RCE") on "B" delay in the *Novartis* appeal. In *Novartis*, the Federal Circuit agreed with the Office that "no ["B" delay] adjustment time is available for any time in continued examination, even if the continued examination was initiated more than three calendar years after the application's filing." *Novartis*, 740 F.3d at 601. However, the *Novartis* court found that if the Office issues a notice of allowance after an RCE is filed, the period after the notice of allowance should not be excluded from the "B" delay period but should be counted as "B" delay. *Id.* at 602. The Federal Circuit issued its mandate in the *Novartis* appeal on March 10, 2014.

Pursuant to the *Novartis* decision, the USPTO has determined that the patentee is entitled to 205 days of "B" delay. In this case, the application was filed May 14, 2009, and the patent issued on September 30, 2014; thus, the application was pending for 1966 days. During this time, the applicant filed an RCE on August 3, 2012. Under 35 U.S.C. § 154(b)(1)(B)(i), the time periods consumed by continued examination ("RCE period") was from August 3, 2012 until the notice of allowance was issued on May 28, 2014 – i.e., 664 days. Subtracting the sum of the RCE period from the total number of days the application was pending results in 1966 – 664 = 1302 days. Thus, for purposes of "B" delay, the application was pending for 1302 – 1097 [i.e., 3 years from the actual filing date] = 205 days beyond the 3-year anniversary of the filing date.

The Office has also determined that no periods of overlap exist.

Overall PTA Calculation

Formula:

"A" delay + "B" delay + "C" delay - Overlap - applicant delay = X

USPTO's Calculation:

652 + 205 + 0 – 0 – 472 = 385

Patentee's Calculation

652 + 205 + 0 – 0 – 472 = 385

Conclusion

Patentee is entitled to PTA of three hundred eighty-five (385) days. Using the formula "A" delay + "B" delay + "C" delay - overlap - applicant delay = X, the amount of PTA is calculated as following: *652 + 205 + 0 – 0 – 472 = 385* days.

Patentee has **two (2) months** from the date of the Office's redetermination of patent term adjustment to request reconsideration of the patent term adjustment if patentee continues to disagree with this determination (no petition fee). This two month period is extendible under 37 CFR 1.136(a). The new/renewed request for reconsideration may be filed without any additional fee. However, patentee who responds more than two months after the mail date of the redetermination is required to pay the extension of time fee. After the period of time to respond has expired, the Office will *sua sponte* issue a certificate of correction adjusting the PTA to three hundred eighty-five (385) days.

Telephone inquiries specific to this decision should be directed to the undersigned Attorney at (571) 272-3212.

/Patricia Faison-Ball/

Patricia Faison-Ball
ATTORNEY ADVISOR
Office of Petitions

Enclosures: Copy of Certificate of Correction
Adjusted PTA calculation

COPY

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT : 8,846,628 B2

DATED : September 30, 2014

INVENTOR(S) : Jeffrey B. Etter

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On the cover page,

[*] Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 USC 154(b) by (260) days

Delete the phrase "by 260 days" and insert – by 385 days--

Patent Term Adjustments

Patent Term Adjustment (PTA) for Application Number: 12/466,213

Filing or 371(c) Date:	05-14-2009	Overlapping Days Between { A and B} or { A and C} :	0
Issue Date of Patent:	09-30-2014	Non-Overlapping USPTO Delays:	732
A Delays:	652	PTO Manual Adjustments:	125
B Delays:	80	Applicant Delays:	472
C Delays:	0	Total PTA Adjustments:	385

Patent Term Adjustment History Explanation of Calculations

Number	Date	Contents Description	PTO(Days)	APPL(Days)	Start
93	06-22-2015	Adjustment of PTA Calculation by PTO	205		0
93	06-22-2015	Adjustment of PTA Calculation by PTO	205		0
92	06-22-2015	Adjustment of PTA Calculation by PTO		80	0
92	06-22-2015	Adjustment of PTA Calculation by PTO		80	0
87.5	08-02-2012	PTA 36 Months	80		0.5
87	09-30-2014	Patent Issue Date Used in PTA Calculation			0
86	08-27-2014	Export to Final Data Capture			0
85	08-26-2014	Dispatch to FDC			0
84	08-25-2014	Application Is Considered Ready for Issue			0
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	05-27-				

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67	05-20-2014	Examiner's Amendment Communication			0
66	05-20-2014	Allowability Notice			0
65	05-12-2014	Information Disclosure Statement considered			0
64	02-27-2014	Affidavit(s) (Rule 131 or 132) or Exhibit(s) Received			0
63	02-28-2014	Date Forwarded to Examiner			0
62	02-27-2014	Response after Non-Final Action		90	51
61	02-27-2014	Request for Extension of Time - Granted			0
60	02-27-2014	Electronic Information Disclosure Statement			0
59	02-27-2014	Information Disclosure Statement (IDS) Filed			0
58	02-03-2014	Mail Interview Summary - Applicant Initiated - Personal			0
57	01-30-2014	Office Action Review			0
56	01-30-2014	Office Action Review			0
55	01-30-2014	Office Action Review			0
54	01-29-2014	Interview Summary - Applicant Initiated - Telephonic			0
53	01-29-2014	Interview Summary- Applicant Initiated			0
52	01-29-2014	Interview Summary - Applicant Initiated - Personal			0
51	08-29-2013	Mail Non-Final Rejection	269		38
50	08-26-2013	Office Action Review			0
49	08-26-2013	Non-Final Rejection			0
48	05-16-2013	Information Disclosure Statement considered			0
47	09-05-2012	Information Disclosure Statement considered			0
46	05-16-2013	Reference capture on IDS			0
45	05-16-2013	Information Disclosure Statement (IDS) Filed		286	38
44	05-16-2013	Information Disclosure Statement (IDS) Filed			0

43	09-05-2012	Reference capture on IDS			0
42	09-05-2012	Information Disclosure Statement (IDS) Filed			0
41	09-05-2012	Information Disclosure Statement (IDS) Filed			0
40	08-06-2012	Date Forwarded to Examiner			0
39	08-03-2012	Amendment Submitted/Entered with Filing of CPA/RCE			0
38	08-03-2012	Request for Continued Examination (RCE)		66	34
37	08-06-2012	Disposal for a RCE / CPA / R129			0
36	08-03-2012	Request for Extension of Time - Granted			0
35	08-03-2012	Workflow - Request for RCE - Begin			0
34	02-28-2012	Mail Final Rejection (PTOL - 326)			0
33	02-27-2012	Office Action Review			0
32	02-24-2012	Final Rejection			0
31	12-01-2011	Information Disclosure Statement considered			0
30	12-01-2011	Reference capture on IDS			0
29	12-02-2011	Date Forwarded to Examiner			0
28	12-01-2011	Response after Non-Final Action		30	25
27	12-01-2011	Request for Extension of Time - Granted			0
26	12-01-2011	Information Disclosure Statement (IDS) Filed			0
25	08-01-2011	Mail Non-Final Rejection	383		0.5
24	07-29-2011	Office Action Review			0
23	07-25-2011	Non-Final Rejection			0
22	04-02-2010	Information Disclosure Statement considered			0
18	01-14-2011	Case Docketed to Examiner in GAU			0
17	04-02-2010	Reference capture on IDS			0
16	04-02-2010	Information Disclosure Statement (IDS) Filed			0
15	04-02-2010	Information Disclosure Statement (IDS) Filed			0
14	11-19-2009	PG-Pub Issue Notification			0
13	08-12-2009	Change in Power of Attorney (May Include Associate POA)			0

12	08-10-2009	Correspondence Address Change	0
11	08-04-2009	Application Dispatched from OIPE	0
10	07-28-2009	Sent to Classification Contractor	0
9	07-28-2009	Filing Receipt - Updated	0
8	07-21-2009	Additional Application Filing Fees	0
7	07-21-2009	A statement by one or more inventors satisfying the requirement under 35 USC 115, Oath of the Applic	0
6	07-21-2009	Applicant has submitted new drawings to correct Corrected Papers problems	0
5	05-28-2009	Filing Receipt	0
4	05-28-2009	Notice Mailed--Application Incomplete--Filing Date Assigned	0
3	05-15-2009	Cleared by OIPE CSR	0
2	05-15-2009	IFW Scan & PACR Auto Security Review	0
1	05-14-2009	Initial Exam Team nn	0
0.5	05-14-2009	Filing date	0

Close Window

Office of Petitions: Routing Sheet

4700

Application No. 12/466,213

This application is being forwarded to your office for further processing. A decision has been rendered on a petition filed in this application, as indicated below. For details of this decision, please see the document PET.OP.DEC filed on the same date as this document.

[X] **GRANTED**

[ ] **DISMISSED**

[ ] **DENIED**

Office of Petitions: Decision Count Sheet

Mailing Month

Application No. 12466213

12466213

For US serial numbers: enter number only, no slashes or commas. Ex: 10123456
For PCT: enter "51+single digit of year of filing+last 5 numbers", Ex. for PCT/US05/12345, enter 51512345

Deciding Official: FAISON-BALL, PATRICIA

Count (1) - Palm Credit 12/466,213

Decision: GRANT

FINANCE WORK NEEDED
☐ Select Check Box for YES

GRANT

Decision Type: 551 - 37 CFR 1.705(d) - PATENT TERM ADJUSTMENT AF

551

Notes:

Count (2)

Decision: n/a

FINANCE WORK NEEDED
☐ Select Check Box for YES

Decision Type: NONE

Notes:

Count (3)

Decision: n/a

FINANCE WORK NEEDED
☐ Select Check Box for YES

Decision Type: NONE

Notes:

Initials of Approving Official (if required)

If more than 3 decisions, attach 2nd count sheet & mark this box ☐

Printed on: *6/22/2015*

Office of Petitions Internal Document - Ver. 5.0

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 8,846,628 B2
APPLICATION NO. : 12/466213
DATED : September 30, 2014
INVENTOR(S) : Etter et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On the Title Page:

The first or sole Notice should read --

Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 385 days.

Signed and Sealed this
Sixth Day of June, 2017

Michelle K. Lee
Director of the United States Patent and Trademark Office

AO 120 (Rev. 08/10)

TO:	REPORT ON THE FILING OR DETERMINATION OF AN ACTION REGARDING A PATENT OR TRADEMARK
Mail Stop 8 Director of the U.S. Patent and Trademark Office P.O. Box 1450 Alexandria, VA 22313-1450	

In Compliance with 35 U.S.C. § 290 and/or 15 U.S.C. § 1116 you are hereby advised that a court action has been filed in the U.S. District Court District of Delaware on the following

☐ Trademarks or ☑ Patents. (☐ the patent action involves 35 U.S.C. § 292.):

DOCKET NO.	DATE FILED	U.S. DISTRICT COURT
	12/22/2021	District of Delaware

PLAINTIFF	DEFENDANT
Celgene Corporation and Celgene International Sàrl	Accord Healthcare Inc.

	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1	US 8,846,628 B2	9/30/2014	Celgene Corporation
2			
3			
4			
5			

In the above—entitled case, the following patent(s)/ trademark(s) have been included:

DATE INCLUDED	INCLUDED BY ☐ Amendment ☐ Answer ☐ Cross Bill ☐ Other Pleading	
PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1		
2		
3		
4		
5		

In the above—entitled case, the following decision has been rendered or judgement issued:

DECISION/JUDGEMENT

CLERK	(BY) DEPUTY CLERK	DATE

Copy 1—Upon initiation of action, mail this copy to Director Copy 3—Upon termination of action, mail this copy to Director
Copy 2—Upon filing document adding patent(s), mail this copy to Director Copy 4—Case file copy