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UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No.	9516-847-999	*Total Pages*	127
First Named Inventor	Jeffrey B. Etter		
Title	ORAL FORMULATIONS OF CYTIDINE ANALOGS AND METHODS OF USE THEREOF		
Express Mail Label No.	**ELECTRONIC FILING**		

APPLICATION ELEMENTS
See MPEP chapters 600 concerning utility patent application contents.

Address to: **Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450**

1. ☒ Fee Transmittal Form
(Submit an original, and a duplicate for fee processing)
[Total Sheets 1]

2. ☐ Applicant claims Small Entity status, see 37 C.F.R. § 1.27

3. ☒ Specification [Total Pages 103]
Both the claims and abstract must start on a new page
(For information on the preferred arrangement, see MPEP 608.01(a)))

4. ☒ Drawing(s) *(35 USC 113)* [Total Sheets 20]

5. ☒ Oath or Declaration [Total Sheets 2]
a. ☒ Unexecuted
b. ☐ A copy from a prior application (37 CFR 1.63(d))
(for continuation/divisional with Box 18 completed)
i. ☐ DELETION OF INVENTOR(S)
Signed statement attached deleting inventor(s) name in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b).

6. ☐ Application Data Sheet. See 37 CFR 1.76 [Total Sheets]

7. ☐ CD-ROM or CD-R in duplicate, large table or Computer Program (Appendix)
☐ Landscape Table on CD

8. ☐ Nucleotide and/or Amino Acid Sequence Submission
(if applicable, items a – c are required)
a. ☐ Computer Readable Form (CRF)
b. ☐ Specification Sequence Listing on:
i. ☐ CD-ROM or CD-R (2 copies); or
ii. ☐ paper
c. ☐ Statement verifying identity of above copies

ACCOMPANYING APPLICATION PARTS

9. ☐ Assignment Papers (cover sheet & document(s))
Name of Assignee

10. ☐ 37 CFR 3.73(b) Statement ☐ Power of Attorney
(when there is an assignee)

11. ☐ English Translation Document *(if applicable)*

12. ☐ Information Disclosure Statement (PTO/SB/08 or PTO-1449)
☐ Copies of citations attached

13. ☐ Preliminary Amendment

14. ☐ Return Receipt Postcard (MPEP 503)
(Should be specifically itemized)

15 ☐ Certified Copy of Priority Document(s)
(if foreign priority is claimed)

16. ☐ Nonpublication Request under 35 U.S.C. 122 (b)(2)(i). Applicant must attach form PTO/SB/35 or it's equivalent

17. ☐ Other:

18. If a CONTINUING APPLICATION, *check appropriate box and supply the requisite information below and in a preliminary amendment, or in an Application Data Sheet under 37 CFR 1.76:*

☐ Continuation ☐ Divisional ☐ Continuation-in-part (CIP) of prior application No.: filed .

Prior application information: Examiner: Group Art Unit:

For CONTINUATION OR DIVISIONAL APPS only: The entire disclosure of the prior application, from which an oath or declaration is supplied under Box 5b, is considered a part of the disclosure of the accompanying continuation or divisional application and is hereby incorporated by reference. The incorporation can only be relied upon when a portion has been inadvertently omitted from the submitted application parts.

19. CORRESPONDENCE ADDRESS

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ATTORNEY DOCKET NO. 9516-847-999 Date: May 14, 2009

Commissioner for Patents
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Sir:

The following utility patent application is enclosed for filing:

Applicant(s): Jeffrey B. Etter
Mei Lai
Jay Thomas Backstrom
Executed on: unexecuted

Title of Invention: ORAL FORMULATIONS OF CYTIDINE ANALOGS AND METHODS OF USE THEREOF

PATENT APPLICATION FEE VALUE

TYPE	NO. FILED	LESS	EXTRA RATE		FEE
Total Claims	91	- 20	71	$52.00 each	$ 3692.00
Independent	4	- 3	1	$220.00 each	$ 220.00
Total Number of Pages w/ drawings (excluding electronically filed sequence or computer code listing)	123	-100	23	$270.00 for each 50 pages over 100	$ 270.00
				Basic Filing Fee	$ 330.00
				Examination Fee ($220.00)	$ 220.00
				Search Fee ($540.00)	$ 540.00
				Multiple Dependency Fee If Applicable ($390.00)	$ 390.00
				Total	$ 5662.00
Applicant qualifies for the 50% Reduction for Independent Inventor, Nonprofit Organization or Small Business Concern.					$ 0.00
Applicant qualifies for an additional $75.00 reduction in Basic Filing Fee for Independent Inventor, Nonprofit Organization or Small Business Concern Filing Electronically.					0.00
				Total Filing Fee	$ 5662.00

[x] Priority of application nos. 61/053,609 filed on May 15, 2008, 61/201,145 filed on December 5, 2008, and 61/157,875 filed on March 5, 2009, is claimed under 35 U.S.C. § 119.

The above calculation is an estimate of the fees due. Please charge the required fees to Jones Day Deposit Account 50-3013.

Respectfully submitted,

for: Anthony M. Insogna 59,239
Andrew V. Trask Reg No.
For: Anthony M. Insogna (Reg No. 35,203)
JONES DAY

Enclosure

This form is not for use with continuation, divisional, re-issue, design or plant patent applications.

NYI-4183304v1

Atty Docket No.
501872-999847/9516-847-999

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.

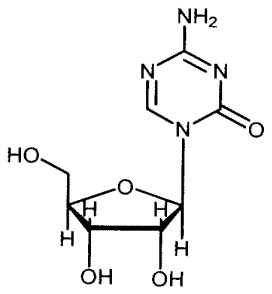
[0006] Primary and secondary MDS are defined by taking into account patients' prior history: previous treatments with chemotherapy, radiotherapy or professional exposure to toxic substances are factors delineating secondary MDS (sMDS) from primary MDS. Cytogenetically, one difference between the two 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: l-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; 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; 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

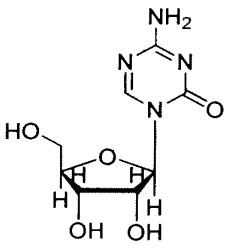
[0075] Provided herein are dosage forms, pharmaceutical formulations and compositions comprising cytidine analogs that release the API substantially in the stomach upon oral administration. In certain embodiments, the cytidine analog is 5-azacytidine. In certain embodiments, the cytidine analog is 5-aza-2'-deoxycytidine (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).

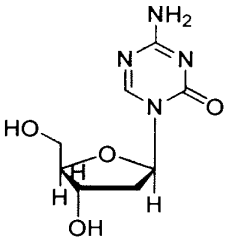
[0076] In certain embodiments, cytidine analogs provided herein include esterified derivatives of cytidine analogs, such as, *e.g.*, esterified derivatives of 5-azacytidine. In particular embodiments, esterified derivatives are cytidine analogs that contain an ester moiety (*e.g.*, an acetyl group) at 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).

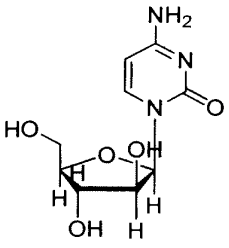
[0077] In certain embodiments, the cytidine analogs provided herein include any compound which is structurally related to cytidine or deoxycytidine and functionally mimics and/or antagonizes the action of cytidine or deoxycytidine. Certain embodiments herein provide salts, cocrystals, solvates (*e.g.*, 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.

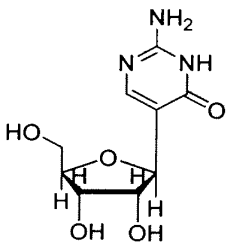
[0078] Cytidine analogs provided herein may be prepared using synthetic methods and procedures referenced herein or otherwise available in the literature. For example, particular methods for synthesizing 5-azacytidine are taught in, *e.g.*, U.S. Patent No. 7,038,038 and references discussed therein, 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.

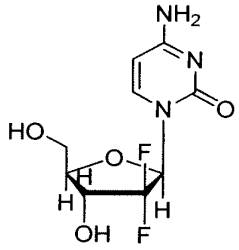
[0079] In certain embodiments, exemplary cytidine analogs have the structures provided below:

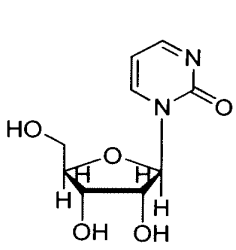

Azacitidine

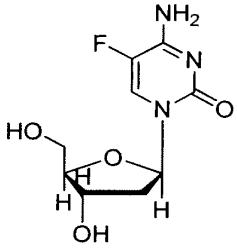

Decitabine

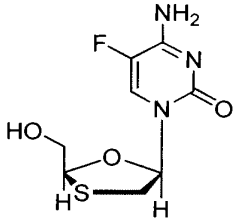

Cytarabine (Ara-C)

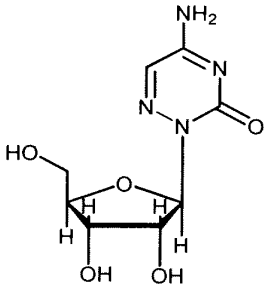

Pseudoisocytidine (psi ICR)

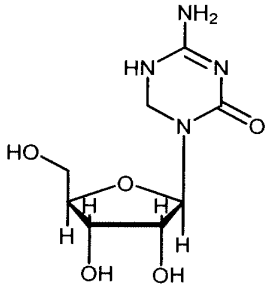

Gemcitabine


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,

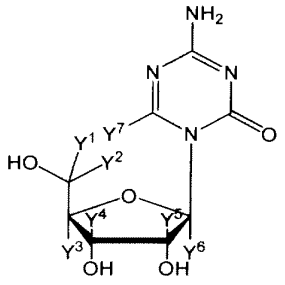
yet it must be ingested in large amounts to pose a significant health risk. As compared with deuterium, a lesser amount of tritium must be consumed before it reaches a hazardous level. Substitution of tritium ("T") for hydrogen results in yet a stronger bond than deuterium and gives numerically larger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

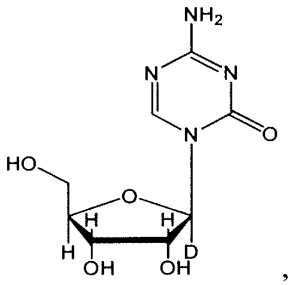
[0089] Certain embodiments herein provide deuterium enriched 5-azacytidine analogs, wherein one or more hydrogen(s) in the 5-azacytidine molecule is/are isotopically enriched with deuterium. In certain embodiments, provided herein are compounds of formula (I):



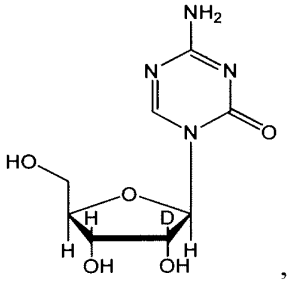
(I),

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).

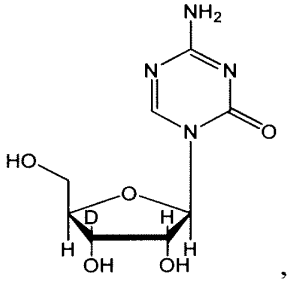
[0090] In certain embodiments, one or more Y atoms on the ribose moiety of Compound (I) are deuterium-enriched. Particular examples include, but are not limited to, the following compounds, in which the label "D" indicates a deuterium-enriched atomic position, *i.e.*, a sample comprising the given compound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



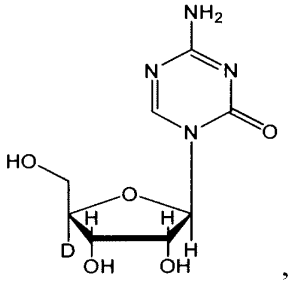
I-1



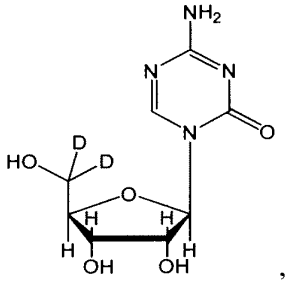
I-2



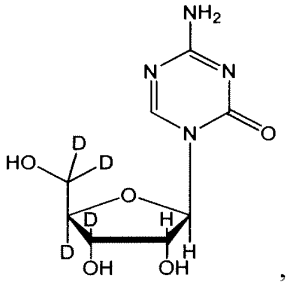
I-3



I-4

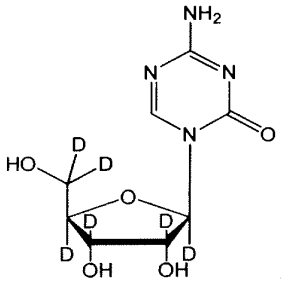


I-5



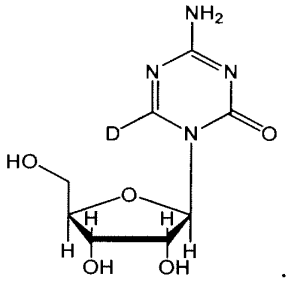
I-6

and



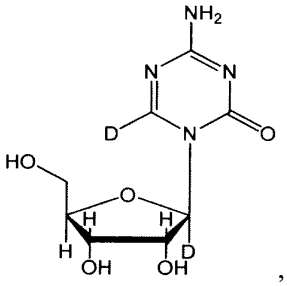
I-7

[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:

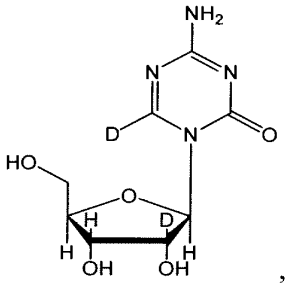


I-8

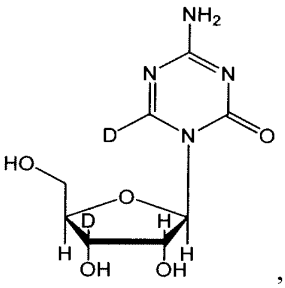
[0092] In certain embodiments, one or more Y atoms on the ribose moiety and the Y atom on the 5-azacytosine moiety of Compound (I) are deuterium-enriched. Particular examples include, but are not limited to, the following compounds, in which the label "D" indicates a deuterium-enriched atomic position,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



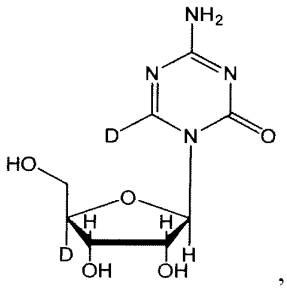
I-9



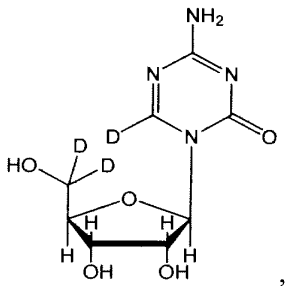
I-10



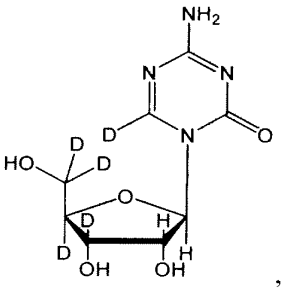
I-11



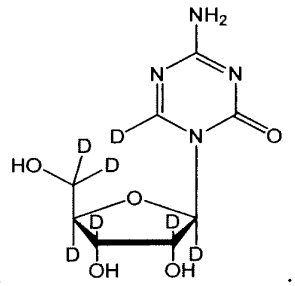
I-12



I-13



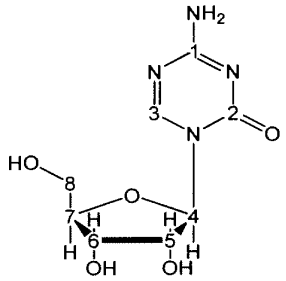
I-14

and  .

I-15

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

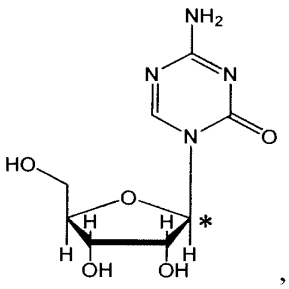
[0094] Certain embodiments herein provide carbon-13 enriched analogs of 5-azacytidine, wherein one or more carbon(s) in the 5-azacytidine molecule is/are isotopically enriched with carbon-13. In certain embodiments, provided herein are compounds of formula (II):



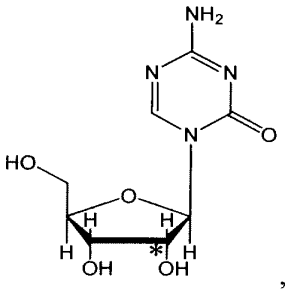
(II),

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.

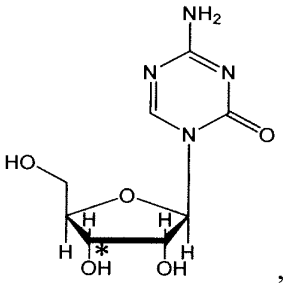
[0095] In certain embodiments, one or more carbon atom(s) of the ribose moiety of Compound (II) are enriched with carbon-13. Particular examples include, but are not limited to, the following compounds, in which the asterisk ("*") indicates a carbon-13 enriched atomic position, *i.e.*, a sample comprising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



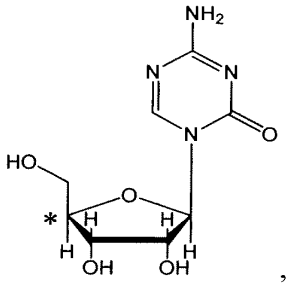
II-1



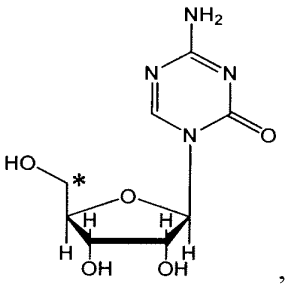
II-2



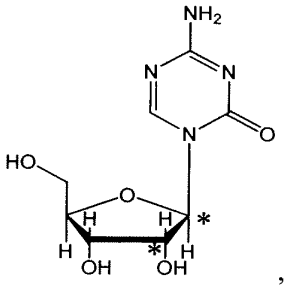
II-3



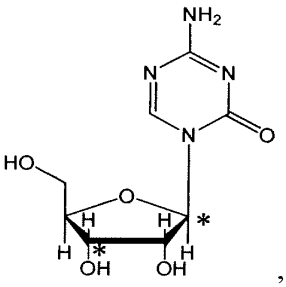
II-4



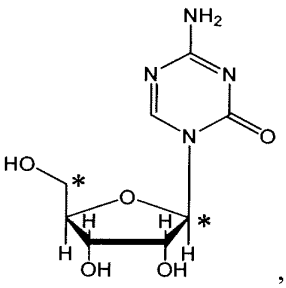
II-5



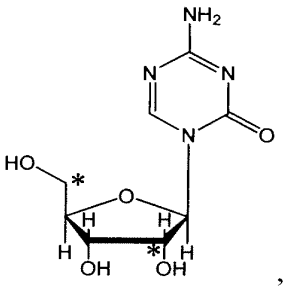
II-6



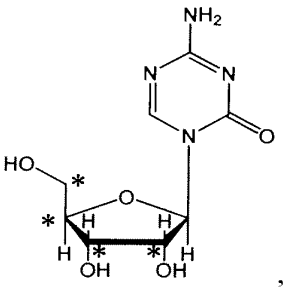
II-7



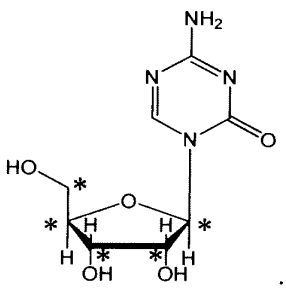
II-8



II-9



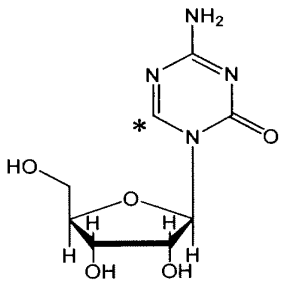
, and



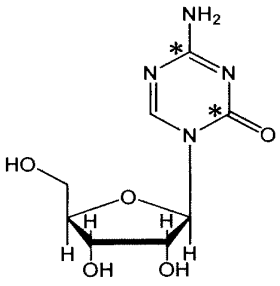
II-10 II-11

[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:

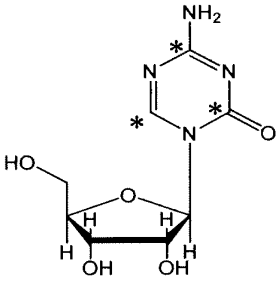


II-12



II-13

, and



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.

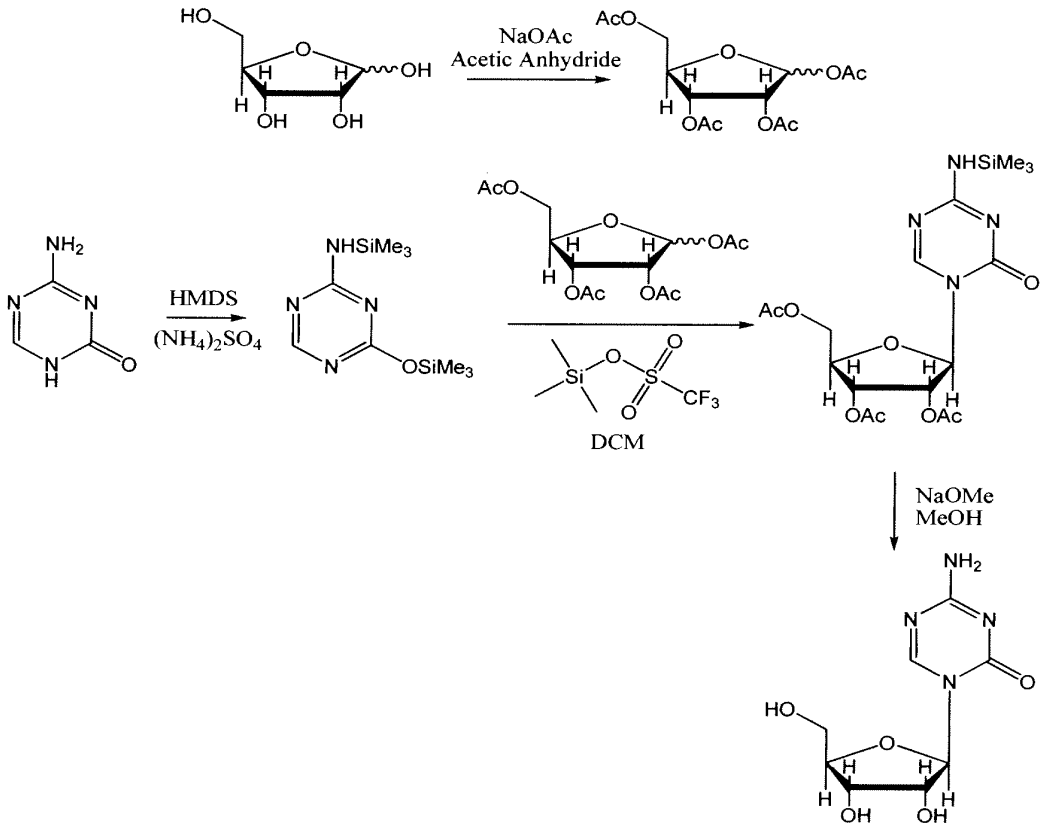
3. Synthesis of Isotopically Enriched Cytidine Analogs

[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.

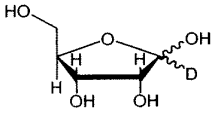
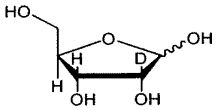
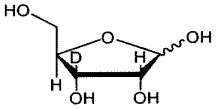
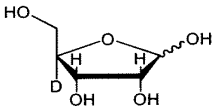
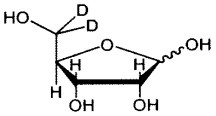
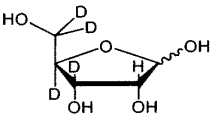
Scheme 1



[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

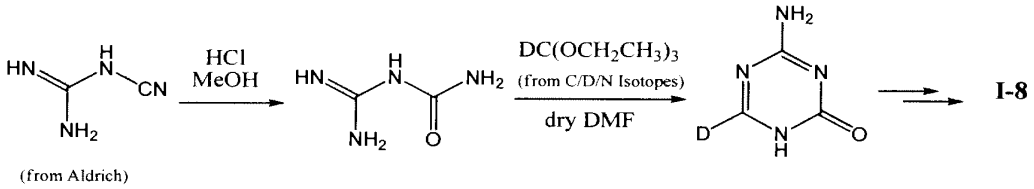
source or prepared following literature procedures. Specific examples of deuterium-enriched ribose starting material include, but are not limited to, the following compounds listed in Table 1, which may be converted to the corresponding deuterium-enriched 5-azacytidine analogs.

TABLE 1

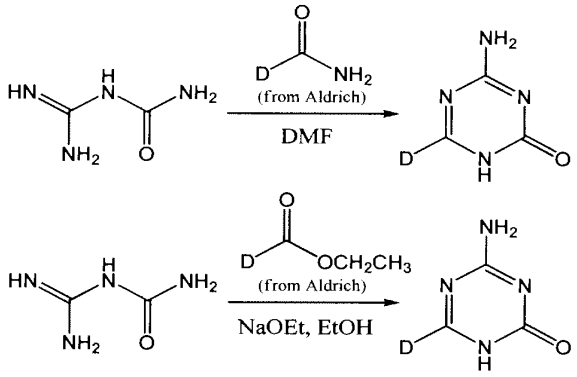
Starting Material	Structure	Source/Reference	5-Azacytidine Product
D-Ribose-1-D		Cambridge Isotope Lab.	I-1
D-Ribose-2-D		Cambridge Isotope Lab.	I-2
D-Ribose-3-D		Omicron Biochemicals, Inc.	I-3
D-Ribose-4-D		Omicron Biochemicals, Inc.	I-4
D-Ribose-5,5'-D_2		Omicron Biochemicals, Inc.	I-5
D-Ribose-3,4,5,5'-D_4		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



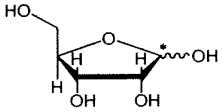
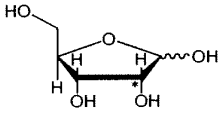
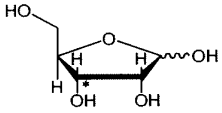
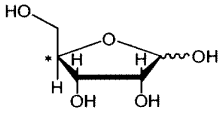
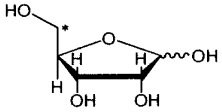
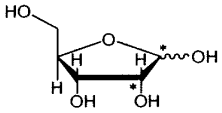
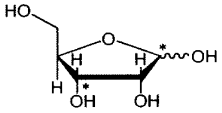
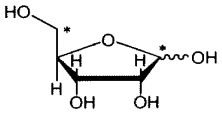
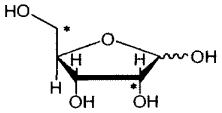
Alternative conditions for preparing 5-azacytosine:

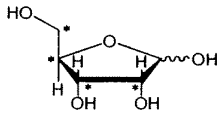
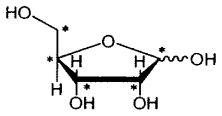


[00103] In other embodiments, both the hydrogen position on the 5-azacytosine ring and one or more hydrogen positions in the ribose portion of 5-azacytidine are enriched with deuterium. Such 5-azacytidine analogs may be prepared, *e.g.*, following Scheme 1, coupling a suitable deuterated ribose starting 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)

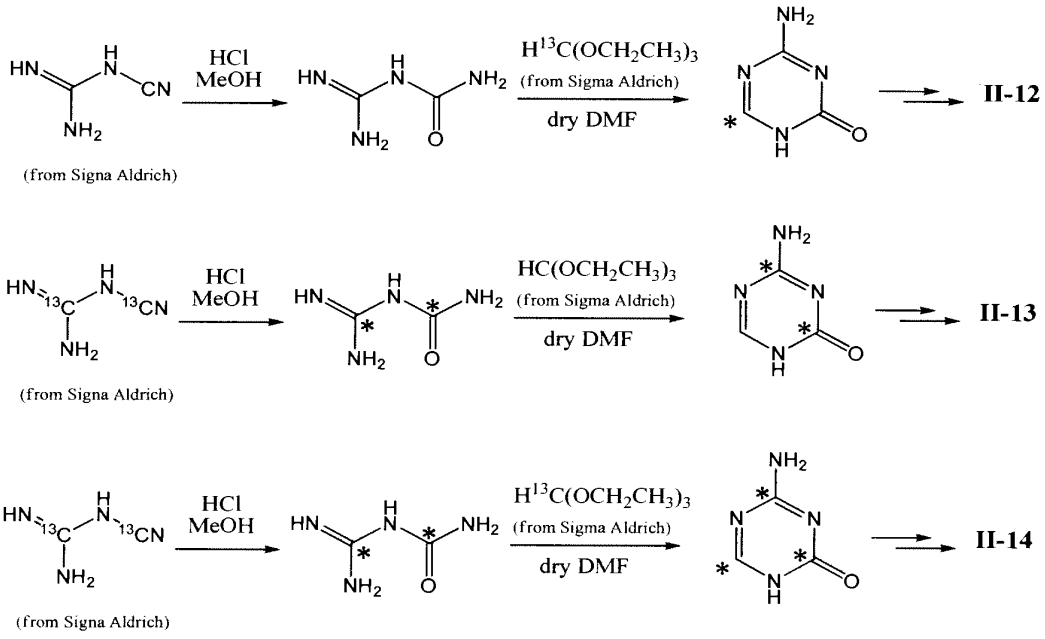
TABLE 2

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 (minimum 99%)	Gattefosse
N,N- Dimethylacetamide		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 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.

24. The method of claim 23, wherein the disease is myelodysplastic syndrome.

25. The method of claim 23, wherein the disease is acute myelogenous leukemia.

26. The method of claim 23, wherein the disease is non-small-cell lung cancer.

27. The method of claim 23, wherein the disease is ovarian cancer.

28. The method of claim 23, wherein the disease is pancreatic cancer.

29. The method of claim 23, wherein the disease is colorectal cancer.

30. The method of claim 23, which results in improved overall survival.

31. The method of claim 23, wherein the method further comprises co-administering to the subject in need thereof an additional therapeutic agent.

32. The method of claim 23, wherein the composition is an immediate release composition.

33. The method of claim 23, wherein the composition further comprises a permeation enhancer.

34. The method of claim 33, wherein the permeation enhancer is d-alpha-tocopheryl polyethylene glycol 1000 succinate.

35. 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. The method of claim 23, wherein the method further comprises not co-administering a cytidine deaminase inhibitor with the cytidine analog.

37. The method of claim 23, wherein the composition is a single unit dosage form.

38. The method of claim 23, wherein the composition is non-enteric-coated.

39. The method of claim 23, wherein the composition is a tablet.

40. The method of claim 23, wherein the composition is a capsule.

41. The method of claim 23, wherein the composition further comprises an excipient selected from mannitol, microcrystalline cellulose, crospovidone, and magnesium stearate.

42. The method of claim 23, wherein the amount of 5-azacytidine is at least about 40 mg.

43. The method of claim 23, wherein the amount of 5-azacytidine is at least about 400 mg.

44. The method of claim 23, wherein the amount of 5-azacytidine is at least about 1000 mg.

45. The method of claim 23, which achieves an area-under-the-curve value of at least about 200 ng-hr/mL following oral administration to the subject.

46. The method of claim 23, which achieves an area-under-the-curve value of at least about 400 ng-hr/mL following oral administration to the subject.

47. The method of claim 23, which achieves a maximum plasma concentration of at least about 100 ng/mL following oral administration to the subject.

48. The method of claim 23, which achieves a maximum plasma concentration of at least about 200 ng/mL following oral administration to the subject.

49. The method of claim 23, which achieves a time to maximum plasma concentration of less than about 180 minutes following oral administration to the subject.

50. The method of claim 23, which achieves a time to maximum plasma concentration of less than about 90 minutes following oral administration to the subject.

51. 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.

52. The pharmaceutical composition of claim 51, wherein the amount of 5-azacytidine is about 40 mg, about 400 mg, or about 1000 mg.

53. The pharmaceutical composition of claim 51, 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.

54. The pharmaceutical composition of claim 51, 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.

55. The pharmaceutical composition of claim 51, 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.

56. The pharmaceutical composition of any one of claims 51 to 55, wherein the composition is prepared in the form of an immediate release composition.

57. The pharmaceutical composition of any one of claims 51 to 55, wherein the composition is prepared for oral administration in combination with an additional therapeutic agent.

58. The pharmaceutical composition of any one of claims 51 to 55, wherein the disease or disorder is myelodysplastic syndrome or acute myelogenous leukemia.

59. The pharmaceutical composition of any one of claims 51 to 55, wherein the composition is a single unit dosage form.

60. The pharmaceutical composition of any one of claims 51 to 55, wherein the composition is a tablet or a capsule.

61. The pharmaceutical composition of any one of claims 51 to 55, wherein the composition further comprises an excipient selected from mannitol, microcrystalline cellulose, crospovidone, and magnesium stearate.

62. 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.

63. The use of claim 62, wherein the disease is myelodysplastic syndrome or acute myelogenous leukemia.

64. The use of claim 62, wherein the amount of 5-azacytidine is about 40 mg, about 400 mg, or about 1000 mg.

65. The use of any one of claims 62 to 64, wherein the composition is prepared for immediate release.

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.

FIG. 1

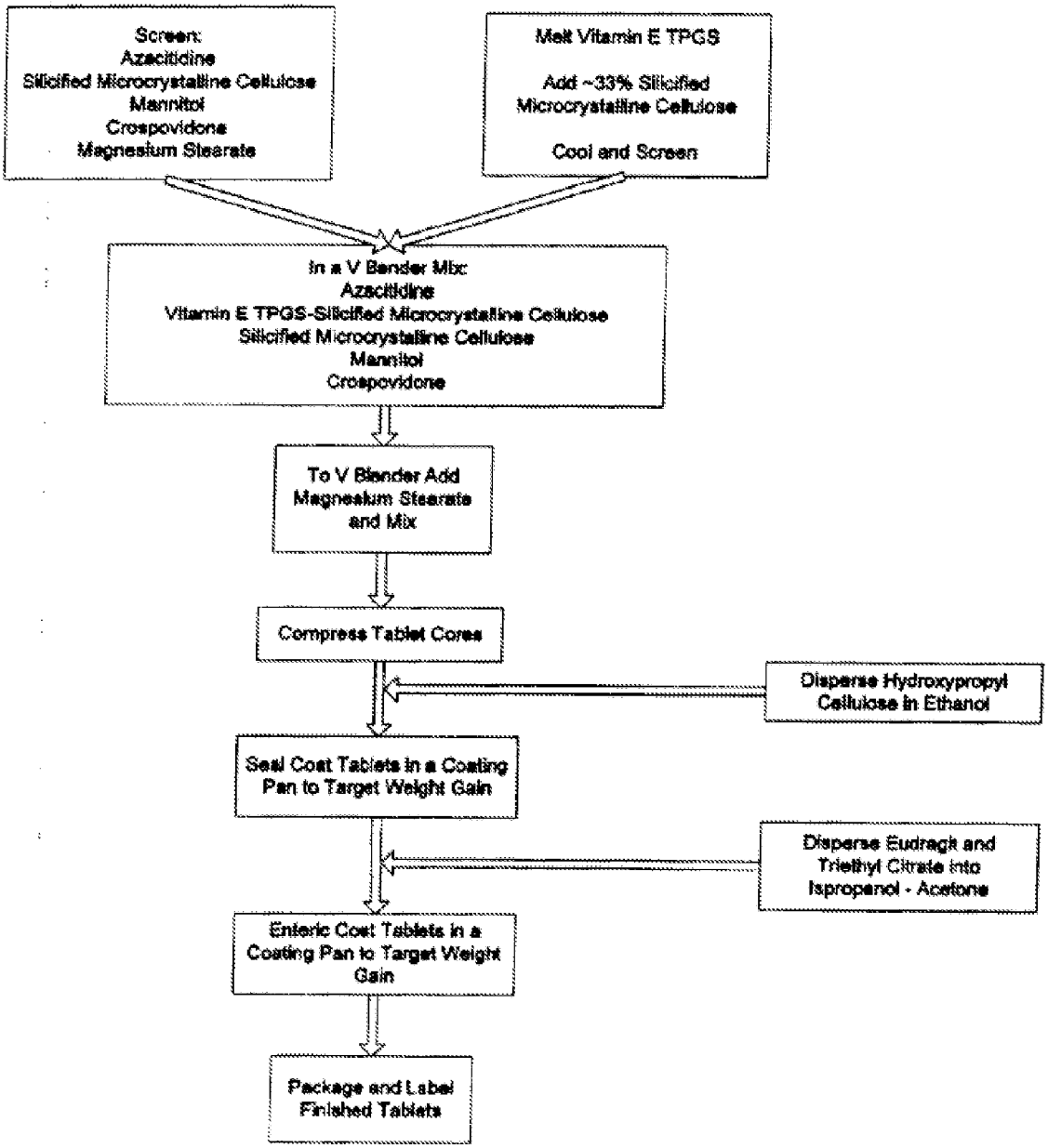

Screen:
Azacitidine
Silicified Microcrystalline Cellulose
Mannitol
Crospovidone
Magnesium Stearate
Melt Vitamin E TPGS
Add ~33% Silicified Microcrystalline Cellulose
Cool and Screen
In a V Bender Mix:
Azacitidine
Vitamin E TPGS-Silicified Microcrystalline Cellulose
Silicified Microcrystalline Cellulose
Mannitol
Crospovidone
To V Blender Add Magnesium Stearate and Mix
Compress Tablet Cores
Disperse Hydroxypropyl Cellulose in Ethanol
Seal Coat Tablets in a Coating Pan to Target Weight Gain
Disperse Eudragit and Triethyl Citrate into Isopropanol - Acetone
Enteric Coat Tablets in a Coating Pan to Target Weight Gain
Package and Label Finished Tablets

FIG. 2

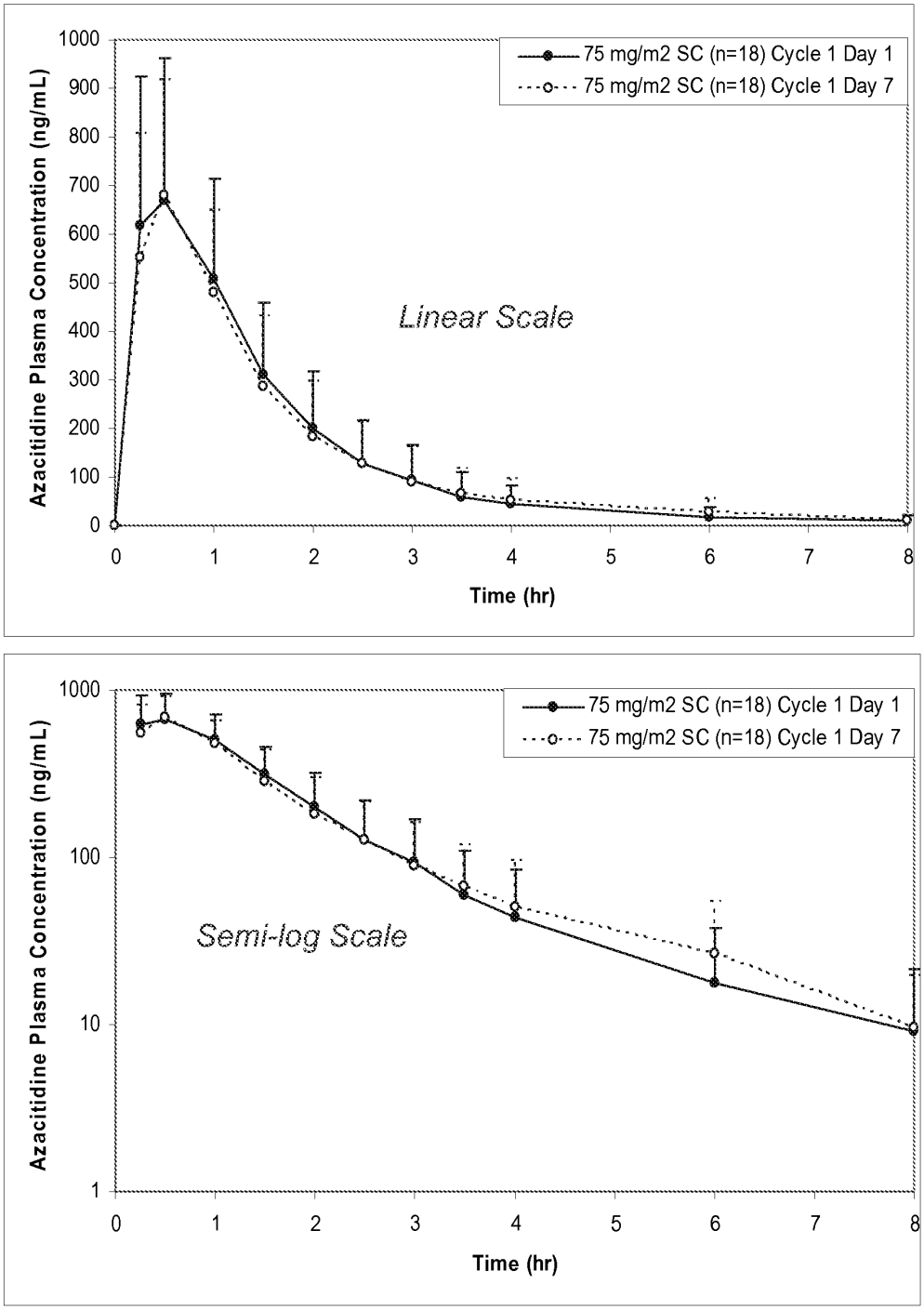
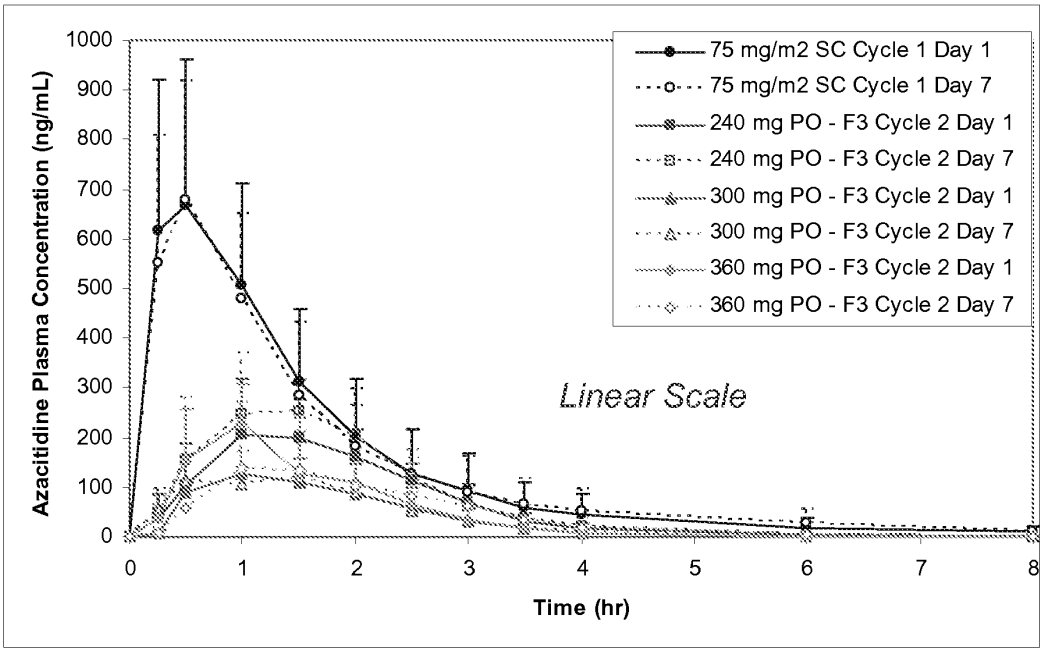


FIG. 3


Azacitidine Plasma Concentration (ng/mL)
1000
900
800
700
600
500
400
300
200
100
0
0
1
2
3
4
5
6
7
8
Time (hr)
75 mg/m2 SC Cycle 1 Day 1
75 mg/m2 SC Cycle 1 Day 7
240 mg PO - F3 Cycle 2 Day 1
240 mg PO - F3 Cycle 2 Day 7
300 mg PO - F3 Cycle 2 Day 1
300 mg PO - F3 Cycle 2 Day 7
360 mg PO - F3 Cycle 2 Day 1
360 mg PO - F3 Cycle 2 Day 7
Linear Scale

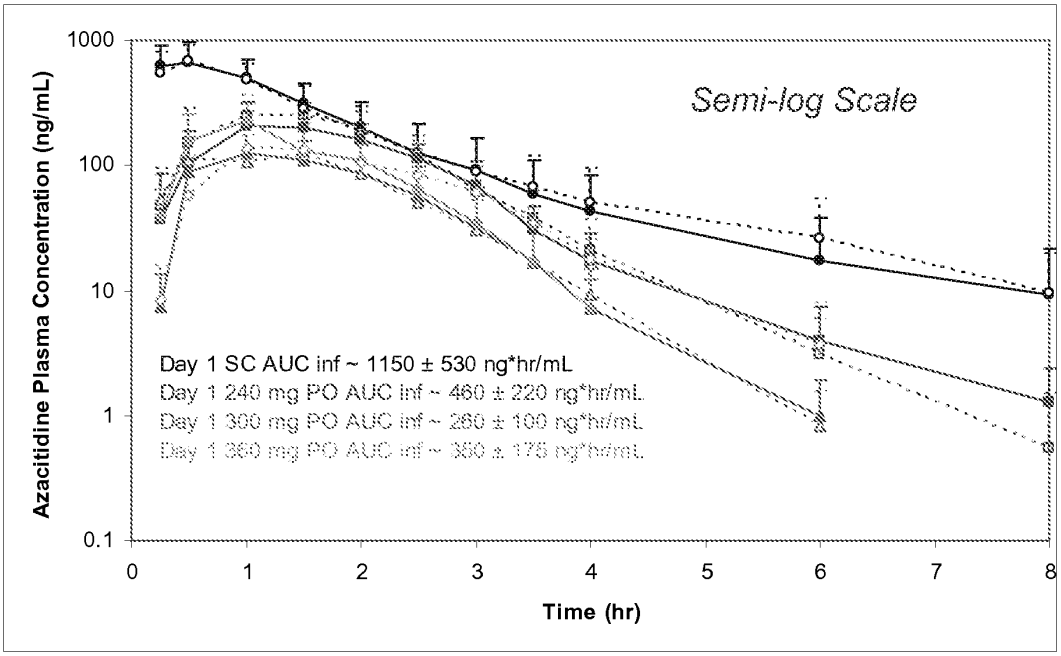

Azacitidine Plasma Concentration (ng/mL)
1000
100
10
1
0.1
0
1
2
3
4
5
6
7
8
Time (hr)
Semi-log Scale
Day 1 SC AUC inf ~ 1150 ± 530 ng*hr/mL
Day 1 240 mg PO AUC inf ~ 460 ± 220 ng*hr/mL
Day 1 300 mg PO AUC inf ~ 260 ± 100 ng*hr/mL
Day 1 360 mg PO AUC inf ~ 360 ± 175 ng*hr/mL

FIG. 4

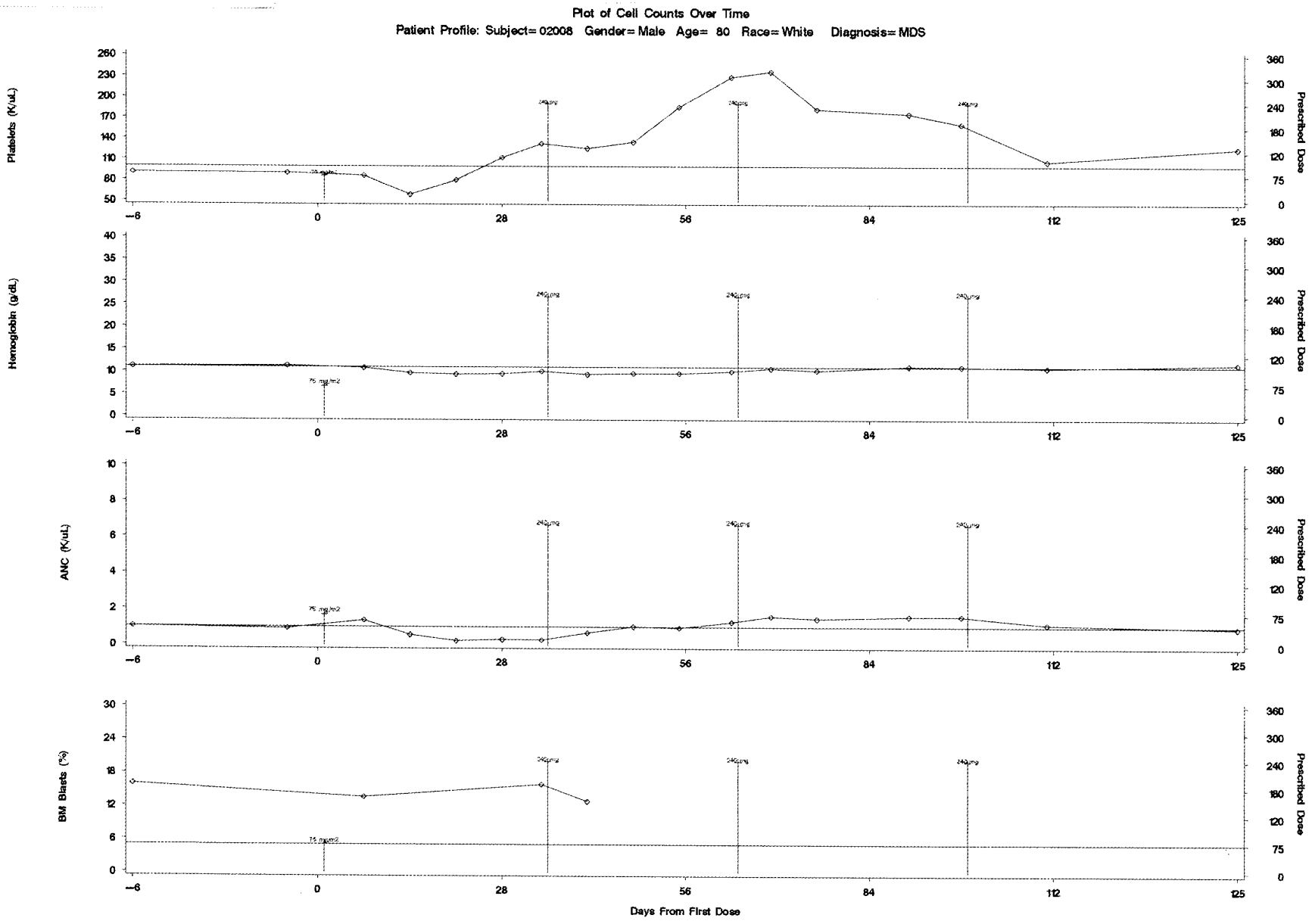


FIG. 5

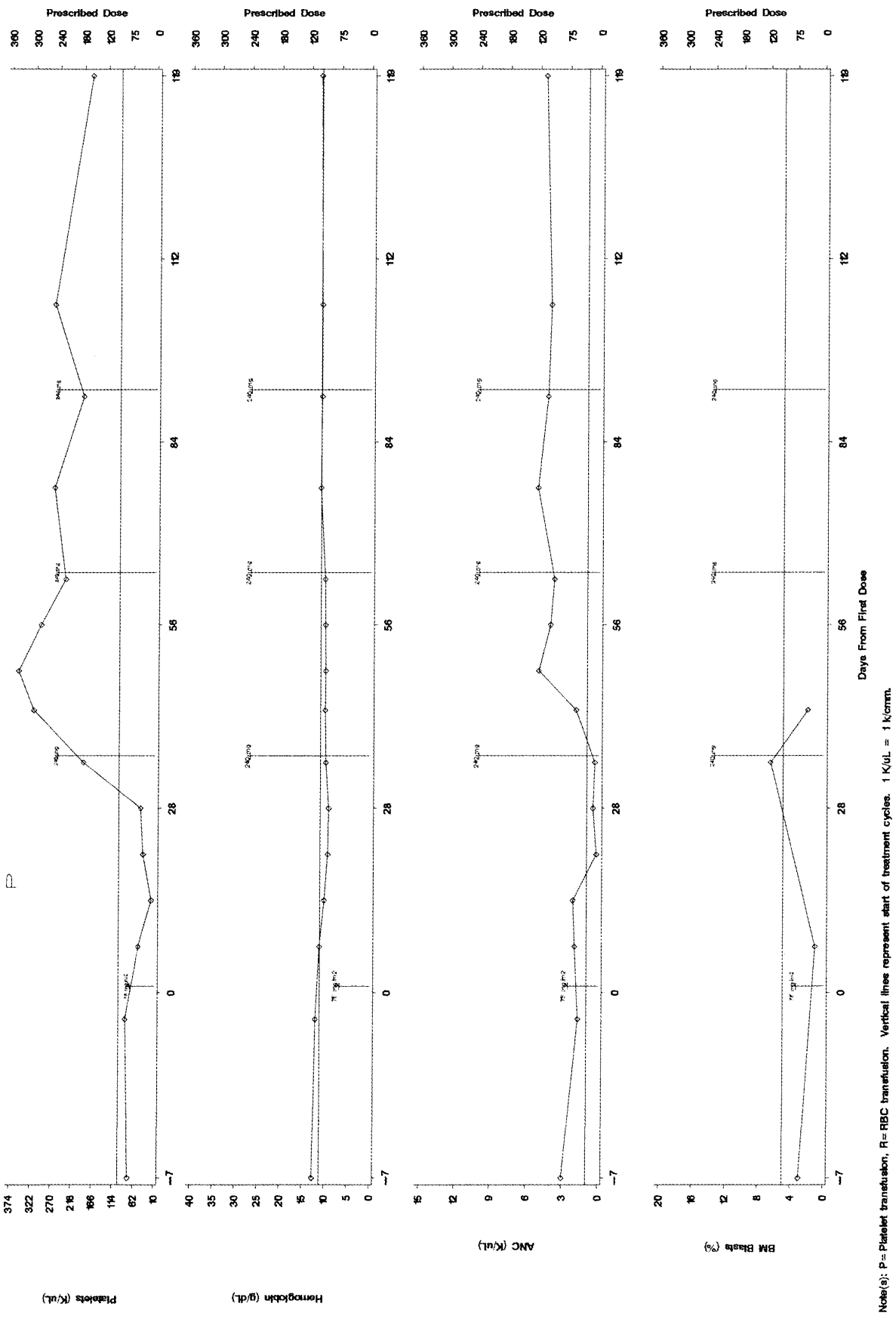


FIG. 6

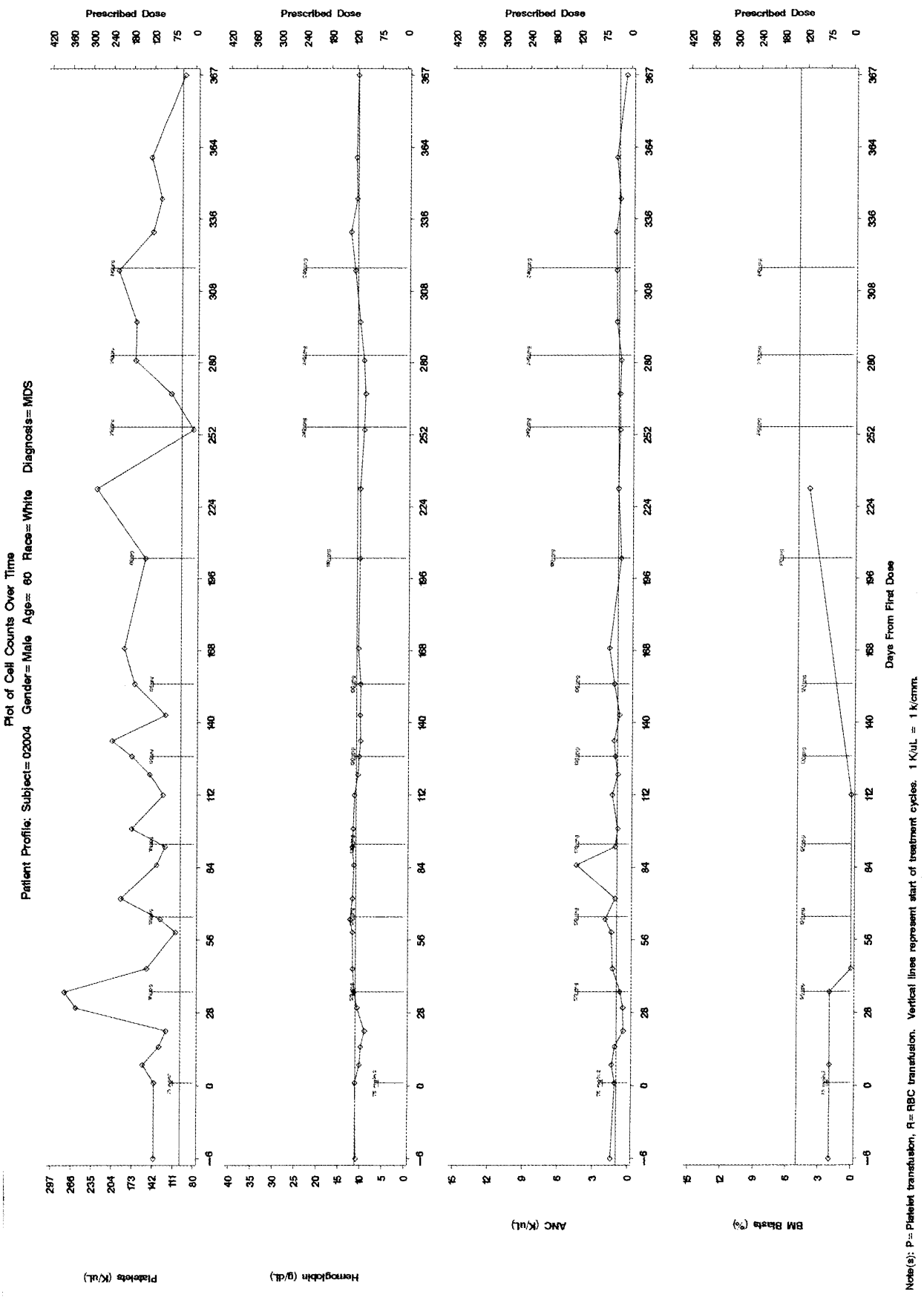


FIG. 7

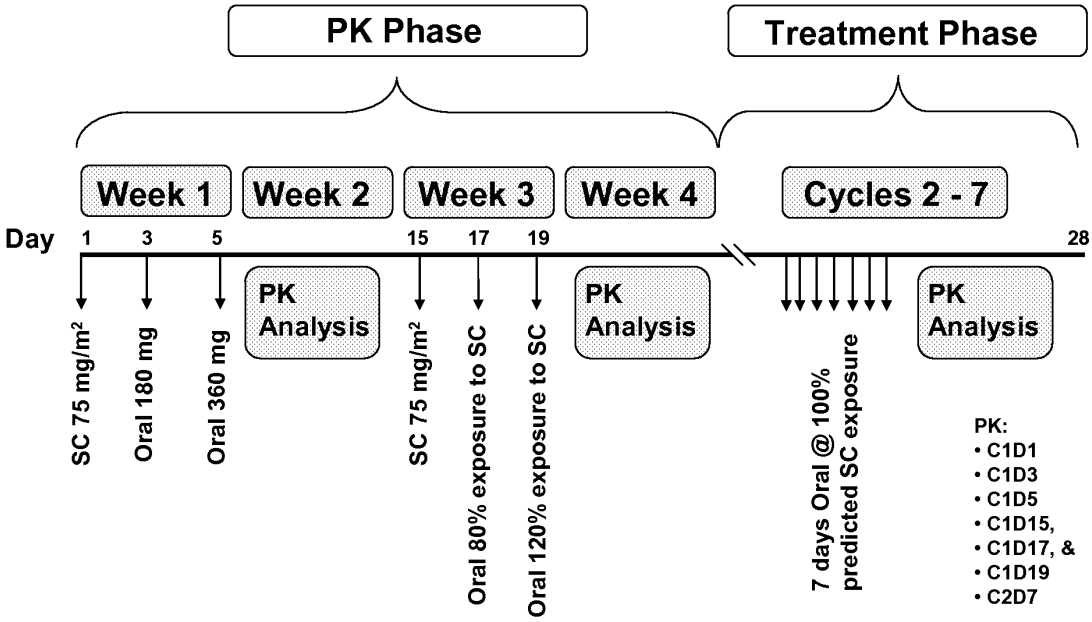


FIG. 8

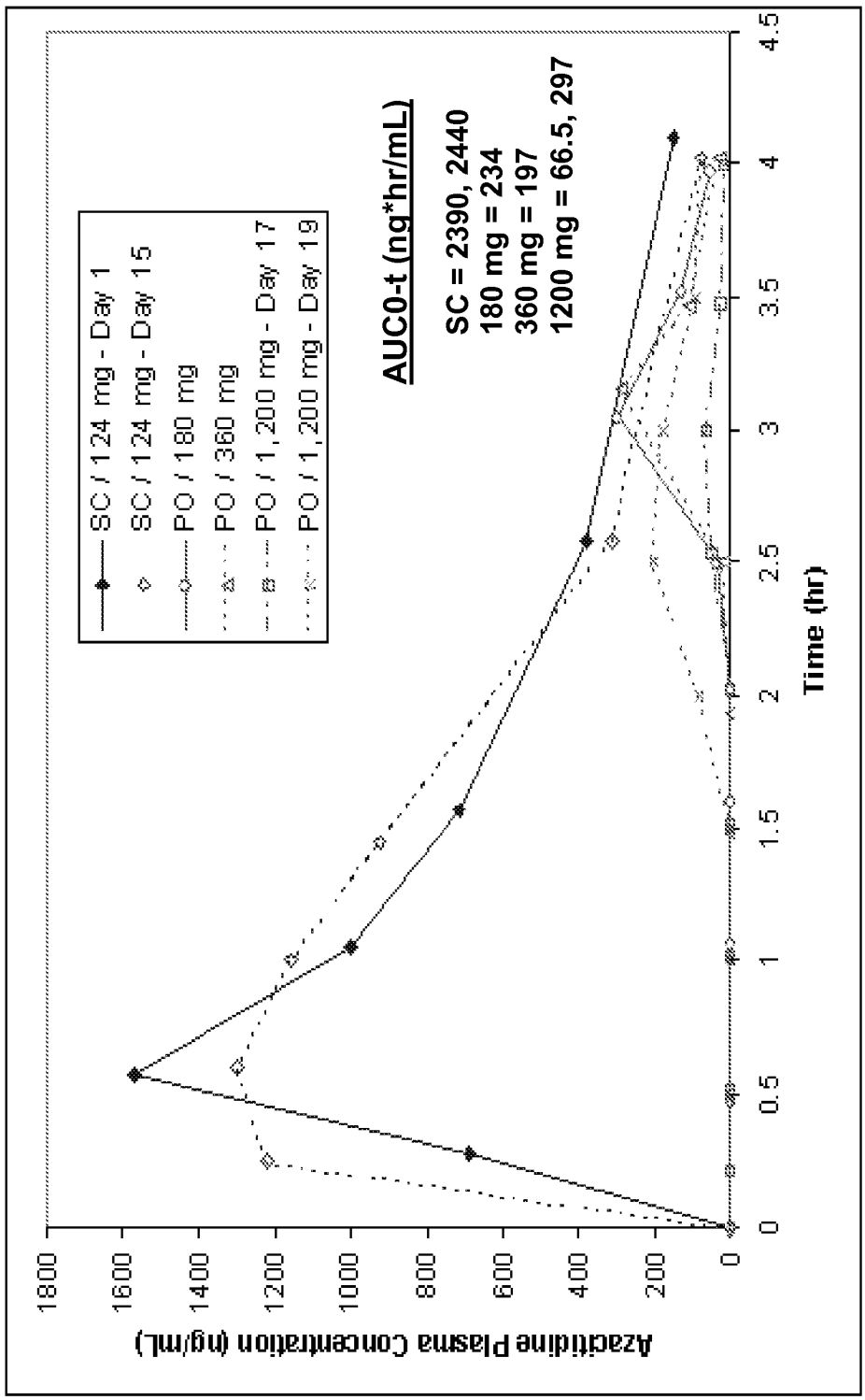


FIG. 9

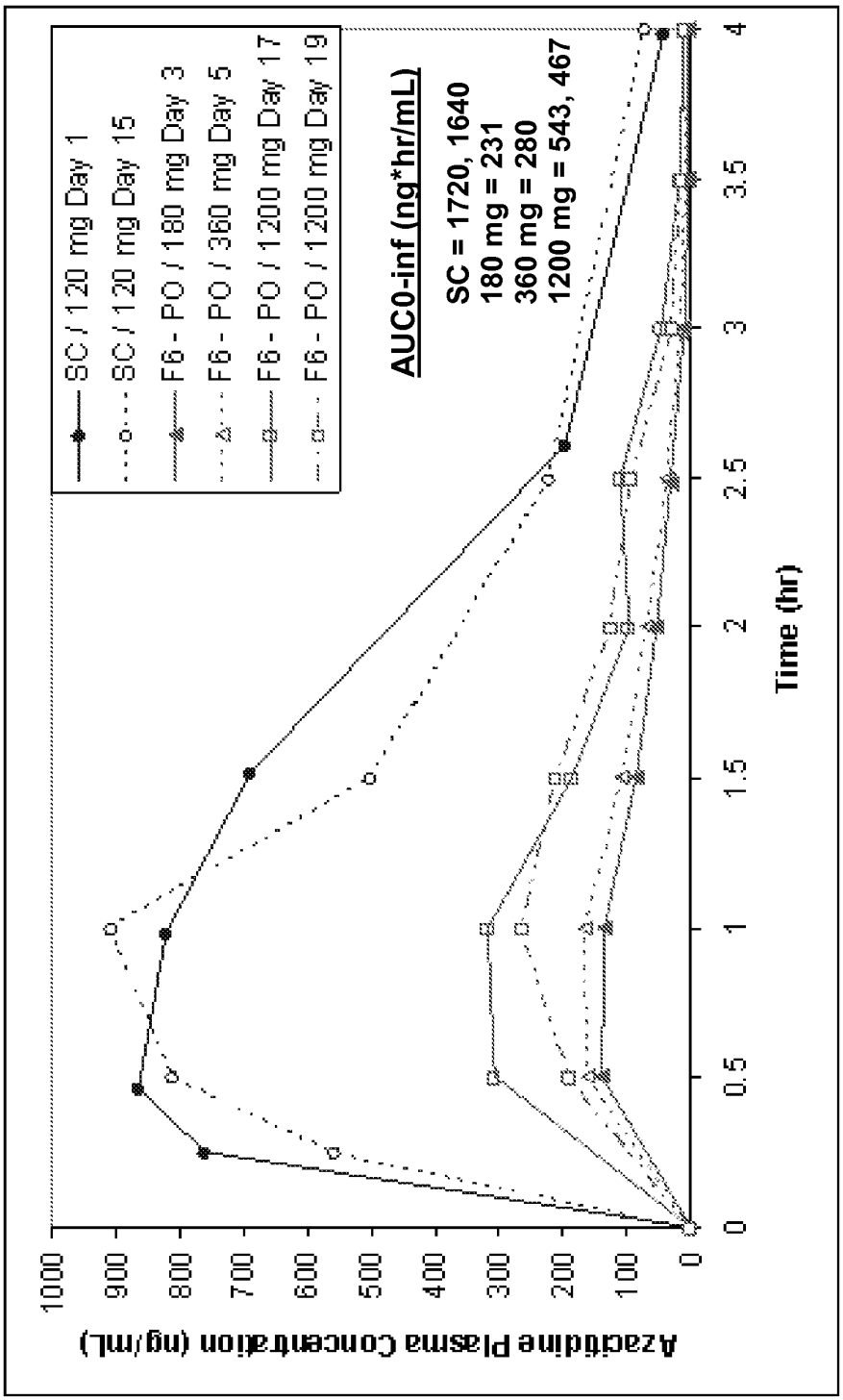


FIG. 10

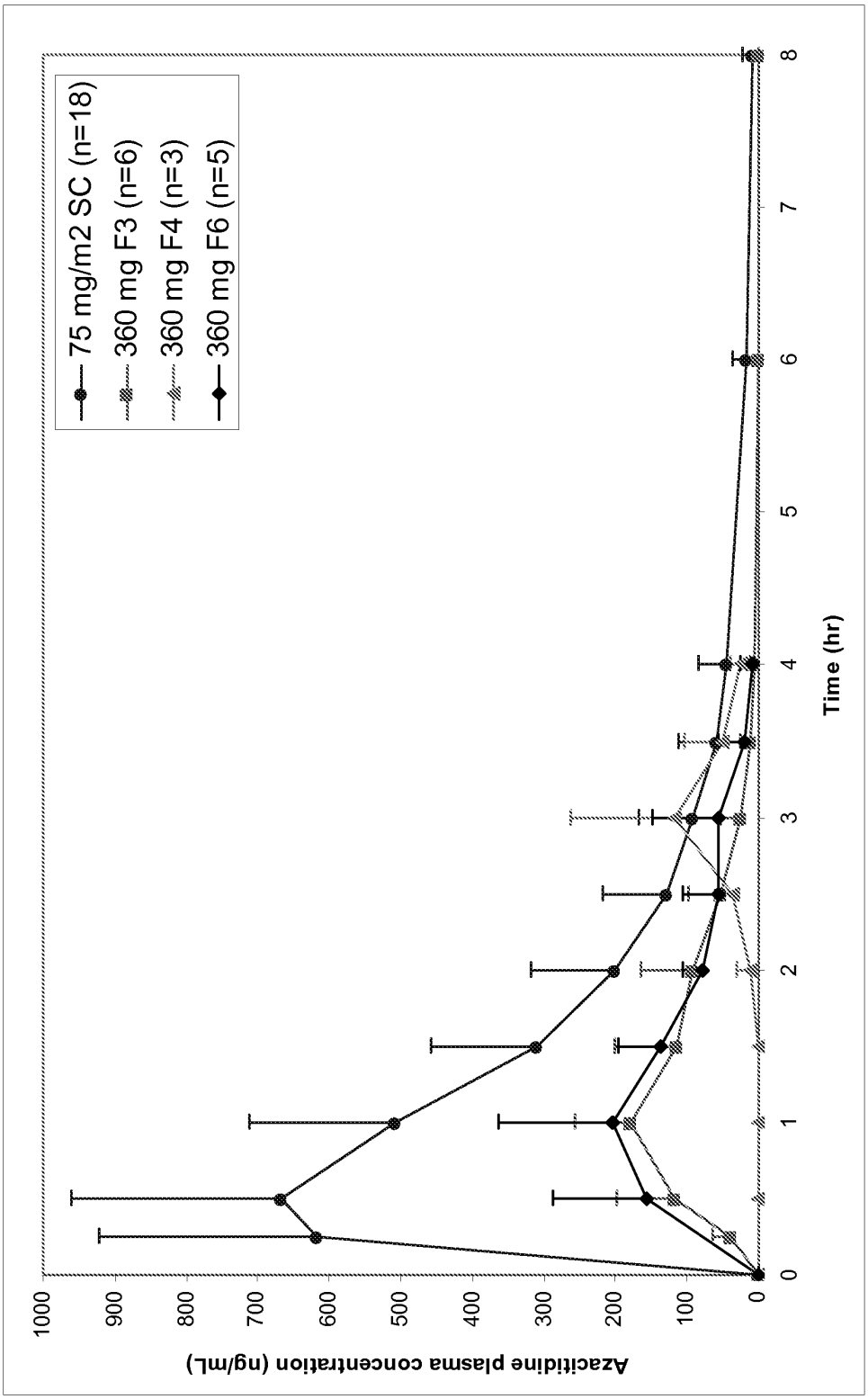


FIG. 11

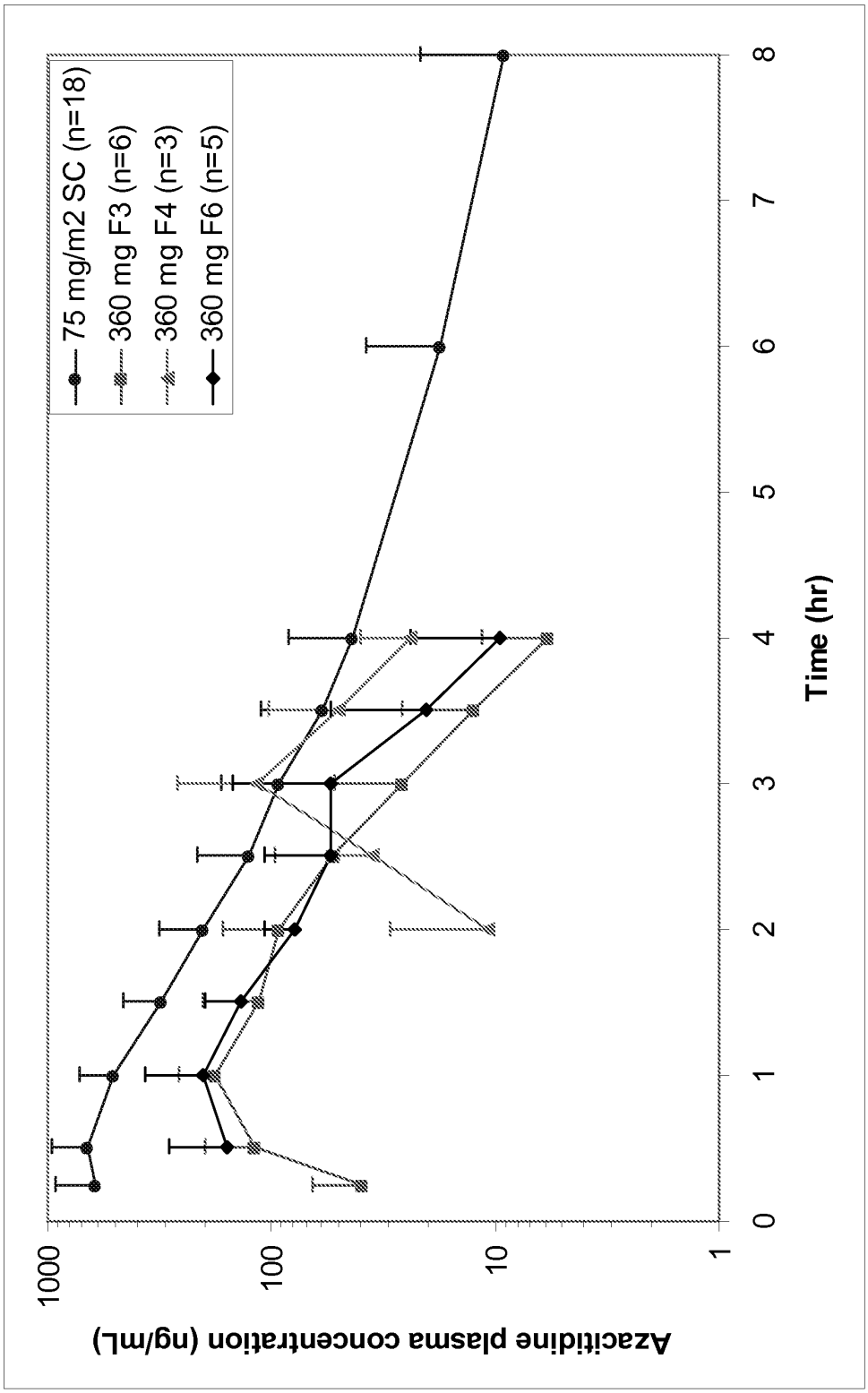


FIG. 12

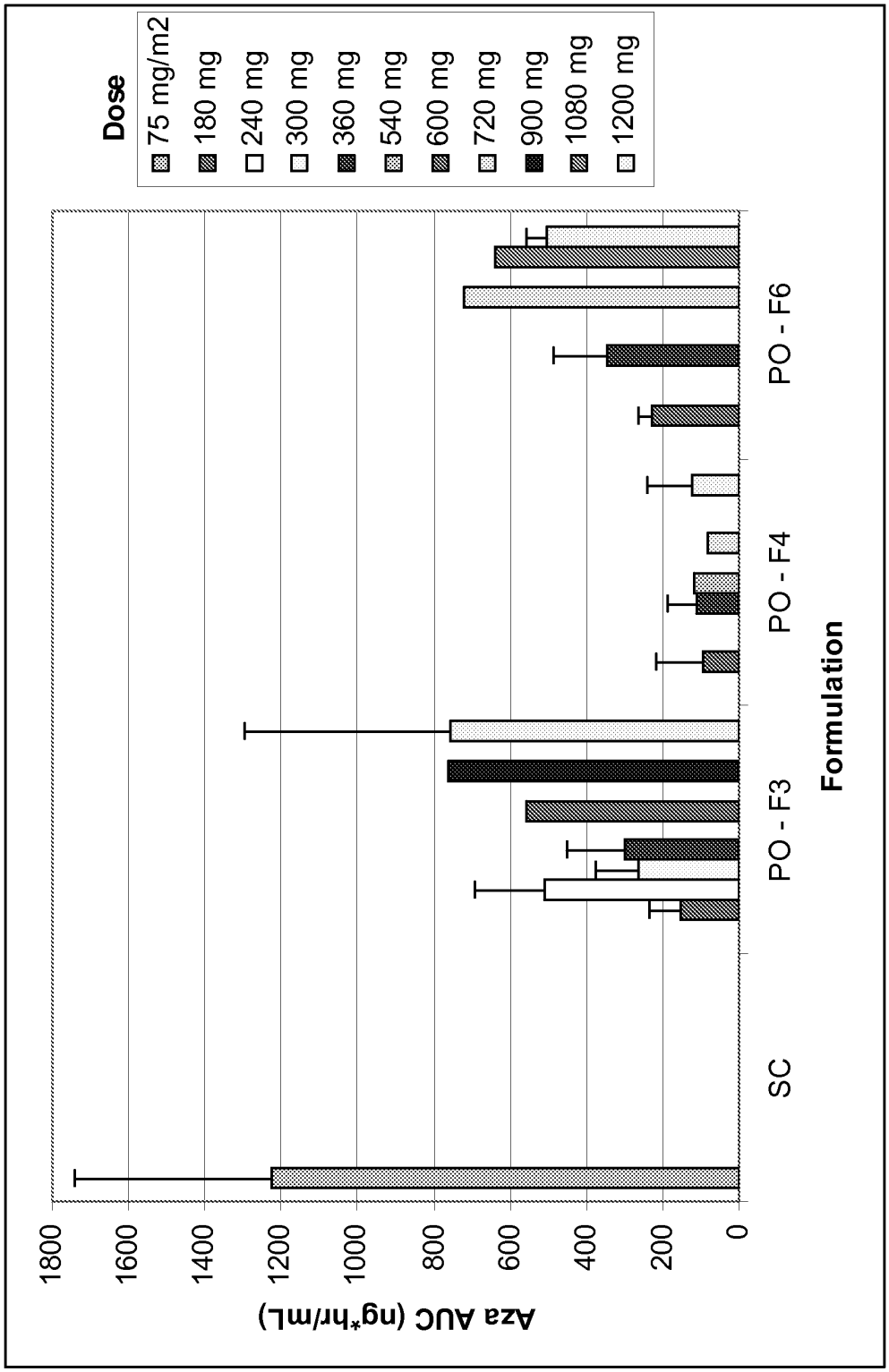


FIG. 13

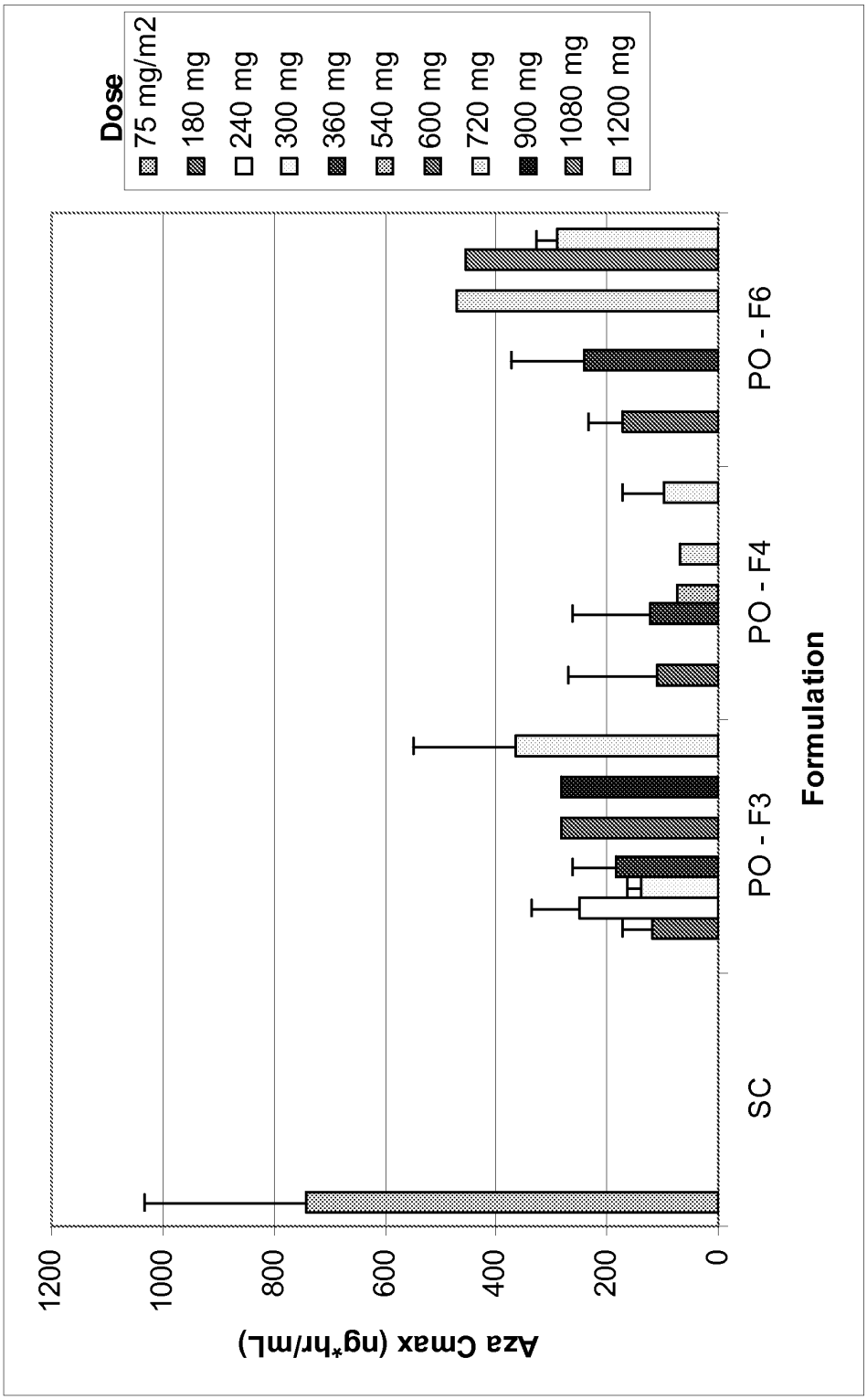


FIG. 14

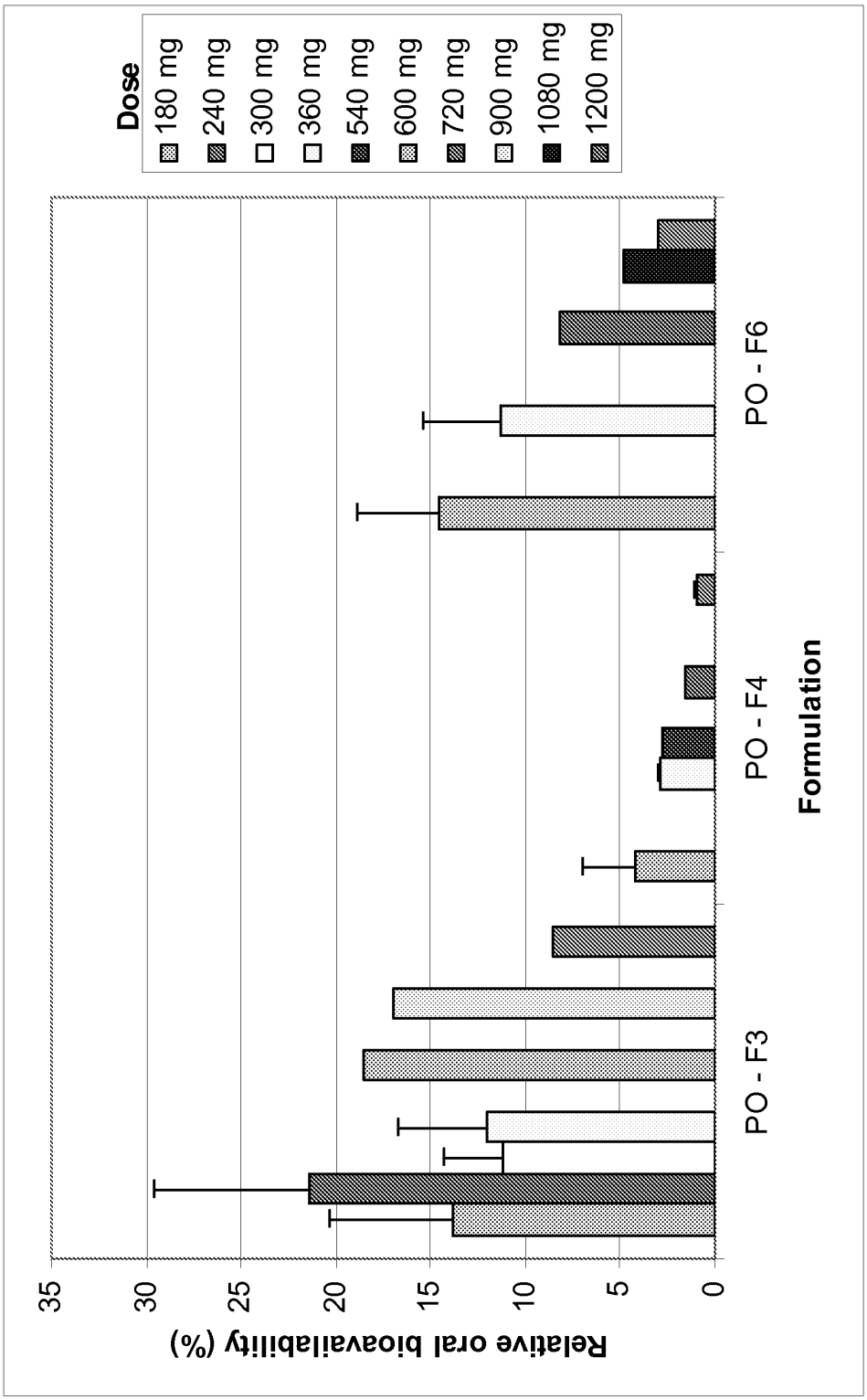


FIG. 15

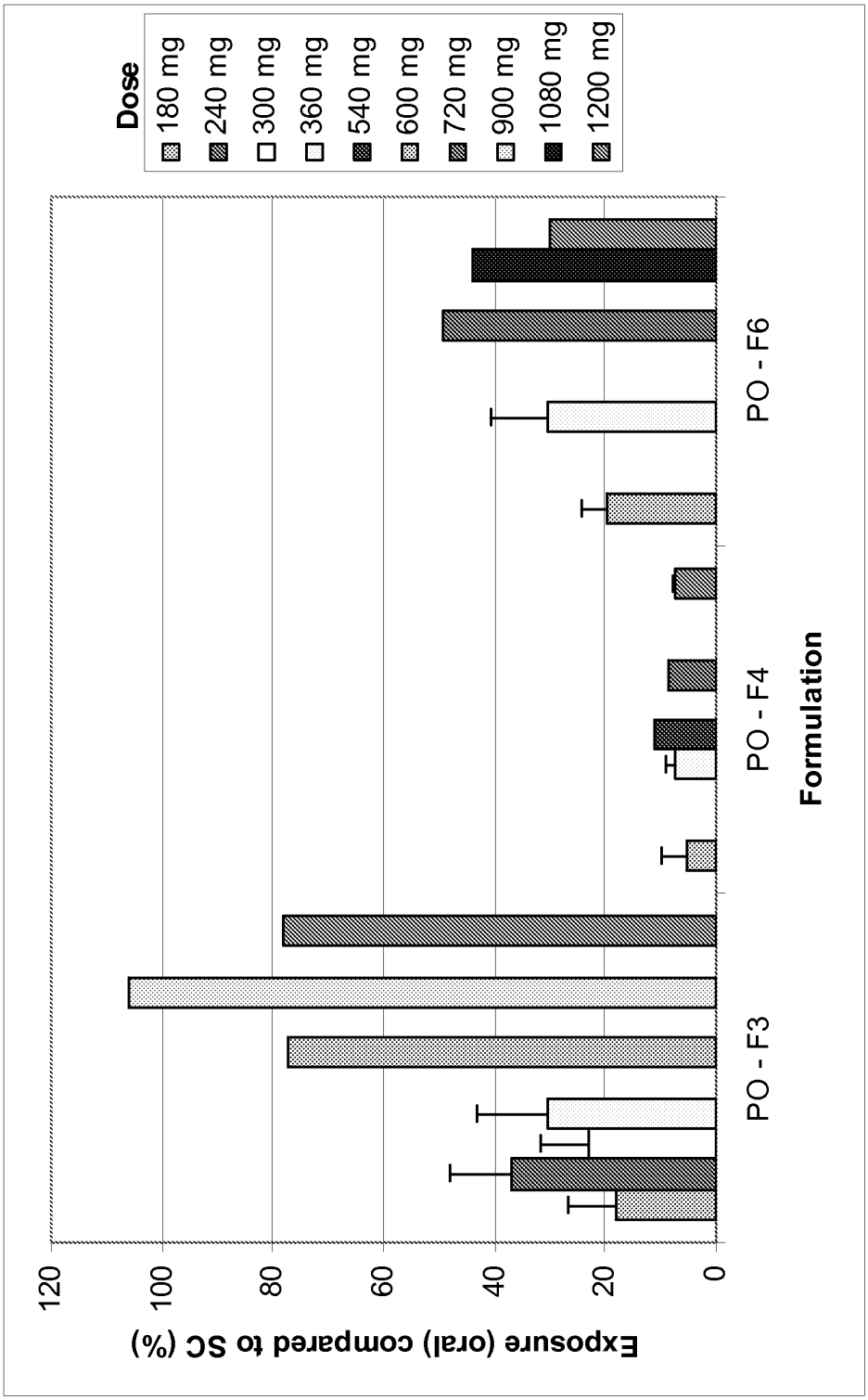


FIG. 16

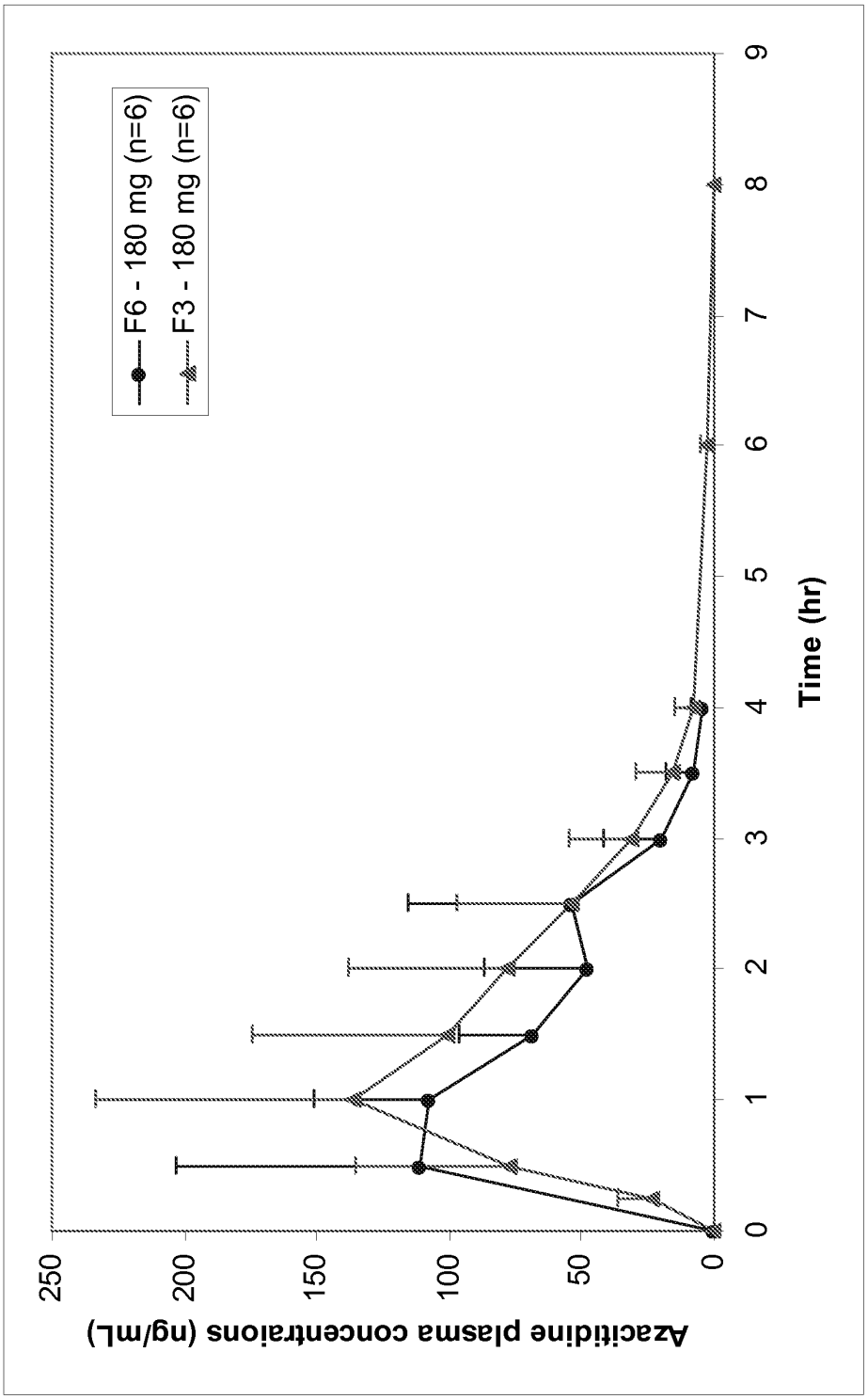


FIG. 17

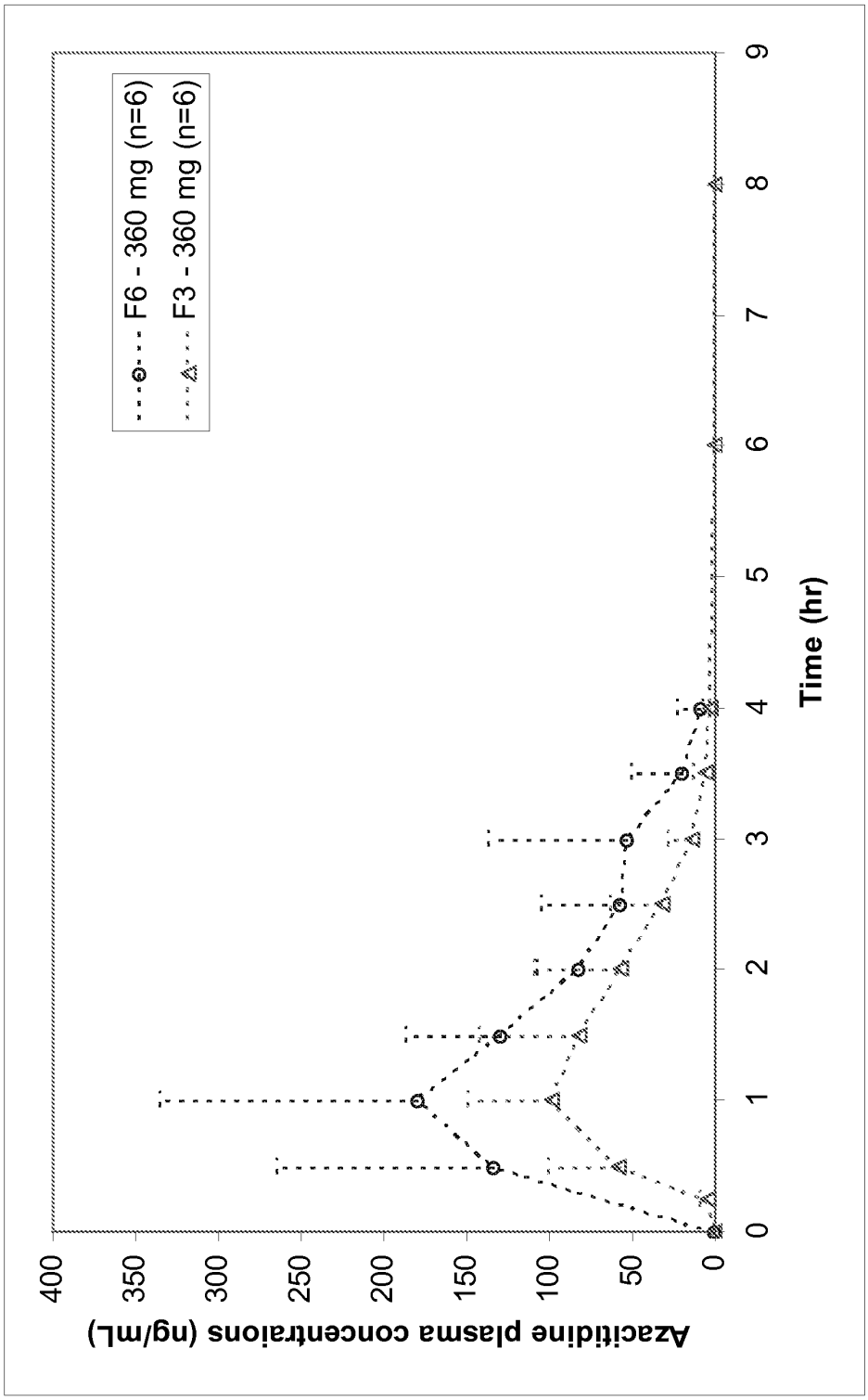


FIG. 18

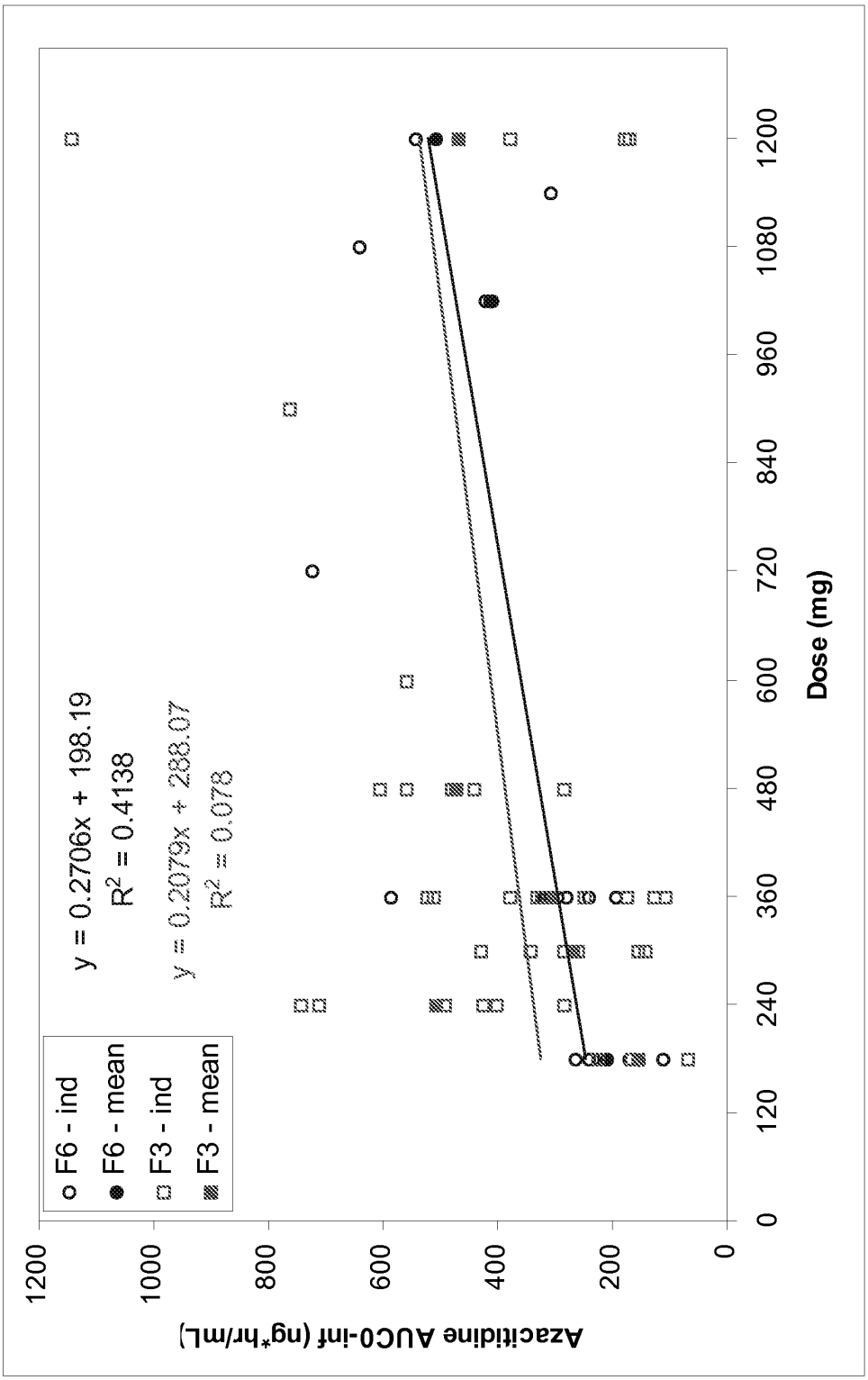


FIG. 19

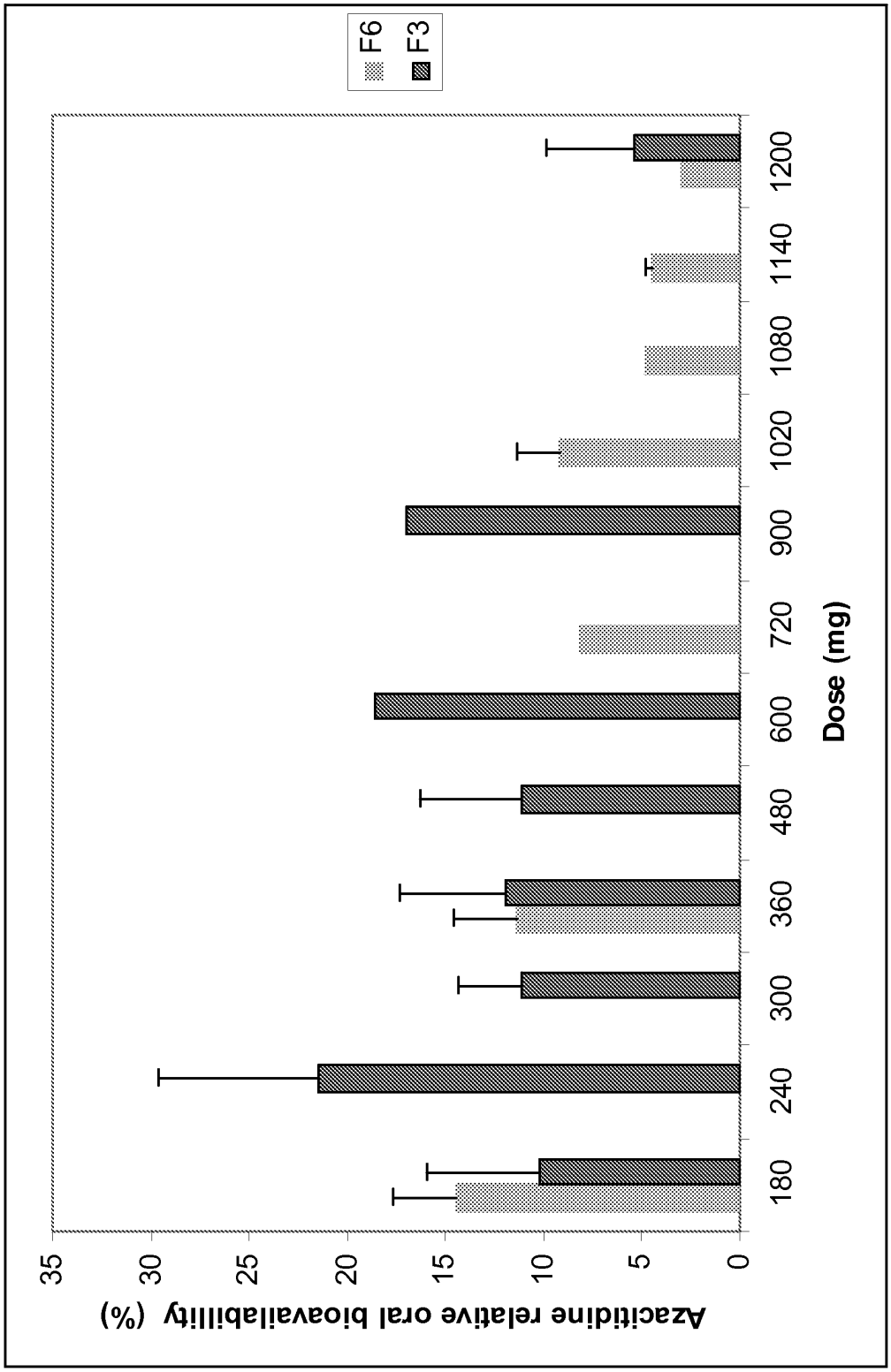
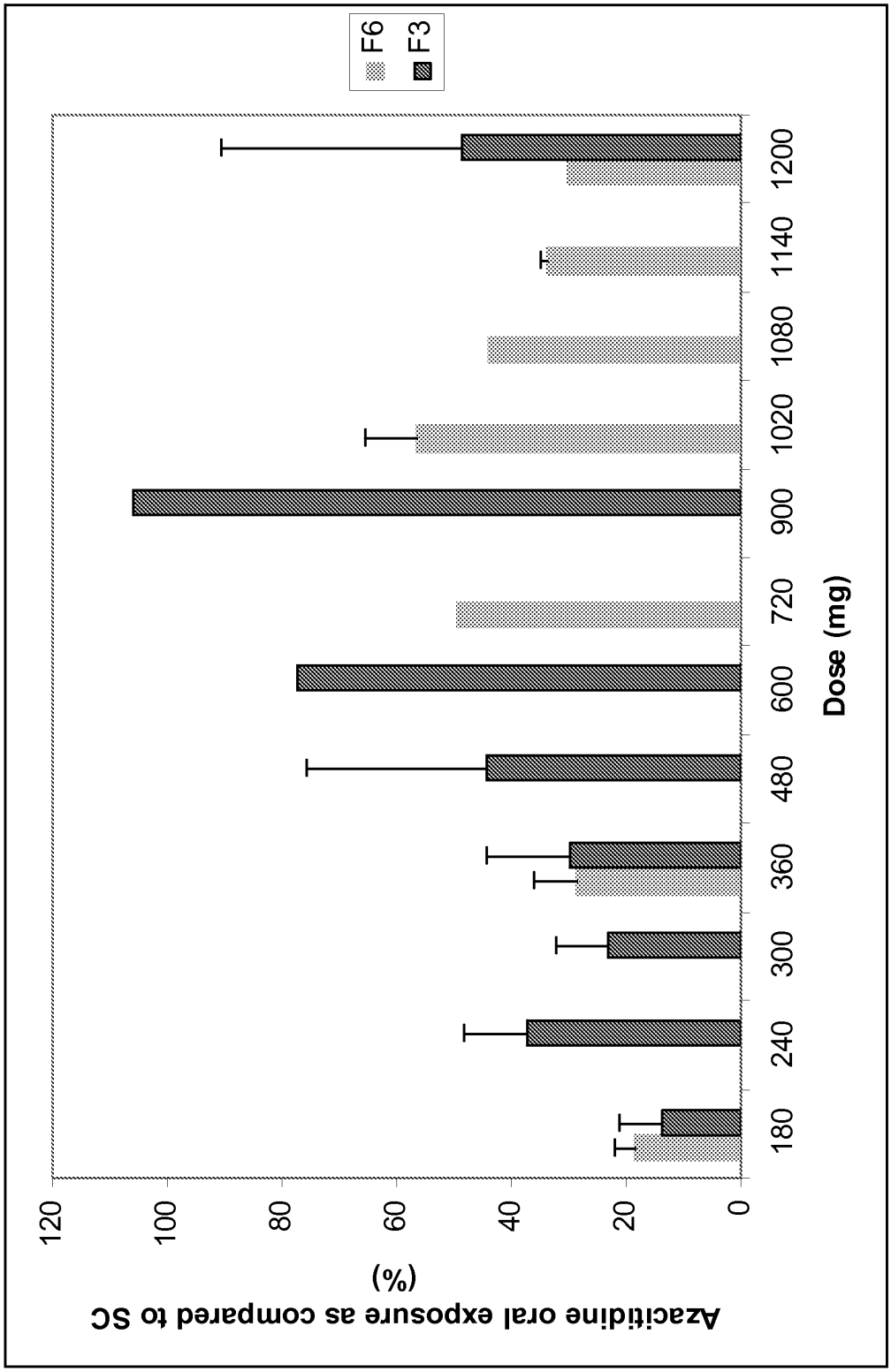


FIG. 20



DECLARATION FOR NON-PROVISIONAL PATENT APPLICATION*

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below at 201 et seq. beneath my name.

I believe I am the original, first and sole inventor if only one name is listed at 201 below, or an original, first and joint inventor if plural names are listed at 201 et seq. below, of the subject matter which is claimed and for which a patent is sought on the invention entitled

ORAL FORMULATIONS OF CYTIDINE ANALOGS AND METHODS OF USE THEREOF

and for which a patent application:

☒ is attached hereto and includes amendment(s) filed on (if applicable)

☐ was filed in the United States on as Application No. (for declaration not accompanying application) with amendment(s) filed on (if applicable)

☐ was filed as PCT international Application No. on and was amended under PCT Article 19 on (if applicable)

I hereby authorize and request the attorneys at Jones Day to insert herein parentheses (Application No. _____ filed _____) the filing date and application number of said application when known.

I hereby state that I have reviewed and understand the contents of the above identified application, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

EARLIEST FOREIGN APPLICATION(S), IF ANY, FILED PRIOR TO THE FILING DATE OF THE APPLICATION				
APPLICATION NUMBER	COUNTRY	DATE OF FILING (day, month, year)	PRIORITY CLAIMED	
			YES ☐	NO ☐
			YES ☐	NO ☐

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below.

PROVISIONAL APPLICATION NUMBER	FILING DATE
61/053,609	May 15, 2008
61/201,145	December 5, 2008
61/157,875	March 5, 2009

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code §112. I acknowledge the duty to disclose information known to me which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

NON-PROVISIONAL APPLICATION SERIAL NO.	FILING DATE	STATUS		
		PATENTED	PENDING	ABANDONED

* For use only when the application is assigned to a company, partnership or other organization.

I hereby declare that all statements made herein of my own knowledge are 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 the application or any patent issuing thereon.

201	FULL NAME OF INVENTOR	LAST NAME: Etter	FIRST NAME: Jeffrey	MIDDLE NAME: B.	
	RESIDENCE & CITIZENSHIP	CITY: Boulder	STATE OR FOREIGN COUNTRY: CO	COUNTRY OF CITIZENSHIP: USA	
	POST OFFICE ADDRESS	STREET: 1318 Deer Trail Road	CITY: Boulder	STATE OR COUNTRY: CO	ZIP CODE: 80302
		SIGNATURE OF INVENTOR 201		DATE	
202	FULL NAME OF INVENTOR	LAST NAME: Lai	FIRST NAME: Mei	MIDDLE NAME:	
	RESIDENCE & CITIZENSHIP	CITY: Longmont	STATE OR FOREIGN COUNTRY: CO	COUNTRY OF CITIZENSHIP: USA	
	POST OFFICE ADDRESS	STREET: 1038 Neon Forest Circle	CITY: Longmont	STATE OR COUNTRY: CO	ZIP CODE: 80504
		SIGNATURE OF INVENTOR 202		DATE	
203	FULL NAME OF INVENTOR	LAST NAME: Backstrom	FIRST NAME: Jay	MIDDLE NAME: Thomas	
	RESIDENCE & CITIZENSHIP	CITY: Leawood	STATE OR FOREIGN COUNTRY: KS	COUNTRY OF CITIZENSHIP: USA	
	POST OFFICE ADDRESS	STREET: 15400 Aberdeen	CITY: Leawood	STATE OR COUNTRY: KS	ZIP CODE: 66224
		SIGNATURE OF INVENTOR 203		DATE	
204	FULL NAME OF INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME	
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP	
	POST OFFICE ADDRESS	STREET	CITY	STATE OR COUNTRY	ZIP CODE
		SIGNATURE OF INVENTOR 204		DATE	
205	FULL NAME OF INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME	
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP	
	POST OFFICE ADDRESS	STREET	CITY	STATE OR COUNTRY	ZIP CODE
		SIGNATURE OF INVENTOR 205		DATE	

Electronic Patent Application Fee Transmittal

Application Number:	
Filing Date:	
Title of Invention:	ORAL FORMULATIONS OF CYTIDINE ANALOGS AND METHODS OF USE THEREOF
First Named Inventor/Applicant Name:	Jeffrey B. Etter
Filer:	Anthony M. Insogna/Rochelle Flowers
Attorney Docket Number:	9516-847-999

Filed as Large Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Utility application filing	1011	1	330	330
Utility Search Fee	1111	1	540	540
Utility Examination Fee	1311	1	220	220
Pages:				
Claims:				
Claims in excess of 20	1202	71	52	3692
Independent claims in excess of 3	1201	1	220	220
Multiple dependent claims	1203	1	390	390

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Miscellaneous:				
		Total in USD ($)		**5392**

Electronic Acknowledgement Receipt

EFS ID:	5338259
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:	Anthony M. Insogna/Rochelle Flowers
Filer Authorized By:	Anthony M. Insogna
Attorney Docket Number:	9516-847-999
Receipt Date:	14-MAY-2009
Filing Date:	
Time Stamp:	18:00:02
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$5392
RAM confirmation Number	13594
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	Transmittal of New Application	Utility_Application_Transmittal.pdf	63255 0fc0ae800d923382873150e517f45d103cdb4347	no	1
Warnings:					
Information:					
2	Transmittal of New Application	Patent_Application_Fee_Transmittal.pdf	37736 db0c12e175b2b104b8b8e514217c94245e73b6f3	no	1
Warnings:					
Information:					
3		Application.pdf	5270116 914998b919fc24f47494cc9e4fc2dd397dbd53f8	yes	103

Multipart Description/PDF files in .zip description

Document Description	Start	End
Specification	1	96
Claims	97	102
Abstract	103	103

Warnings:					
Information:					
4	Drawings-only black and white line drawings	Drawings.pdf	762874 72b4a6df1f6ef0482dc579c19fb1560e5846fa5f	no	20
Warnings:					
Information:					
5	Oath or Declaration filed	Declaration_unexecuted.pdf	99923 8fd5d8ddd463ab44eb0d1dbce476a39eb6aedbab	no	2
Warnings:					
Information:					
6	Fee Worksheet (PTO-875)	fee-info.pdf	39685 ec4f658ee59689fb769c40a395cf976d4cca52af	no	2
Warnings:					

Information:	
Total Files Size (in bytes):	6273589

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.

DocCode - SCORE

SCORE Placeholder Sheet for IFW Content

Application Number: 12466213 Document Date: 5/14/2009

The presence of this form in the IFW record indicates that the following document type was received in electronic format on the date identified above. This content is stored in the SCORE database.

- Drawings – Other than Black and White Line Drawings

Since this was an electronic submission, there is no physical artifact folder, no artifact folder is recorded in PALM, and no paper documents or physical media exist. The TIFF images in the IFW record were created from the original documents that are stored in SCORE.

To access the documents in the SCORE database, refer to instructions developed by SIRA.

At the time of document entry (noted above):

- Examiners may access SCORE content via the eDAN interface.
- Other USPTO employees can bookmark the current SCORE URL (http://es/ScoreAccessWeb/).
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Form Revision Date: February 8, 2006

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United States Patent and Trademark Office
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Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
12/466,213	05/14/2009	Jeffrey B. Etter	9516-847-999

CONFIRMATION NO. 5370

20583
JONES DAY
222 EAST 41ST ST
NEW YORK, NY 10017

FORMALITIES LETTER

OC000000036112151

Date Mailed: 05/28/2009

NOTICE TO FILE MISSING PARTS OF NONPROVISIONAL APPLICATION

FILED UNDER 37 CFR 1.53(b)

Filing Date Granted

Items Required To Avoid Abandonment:

An application number and filing date have been accorded to this application. The item(s) indicated below, however, are missing. Applicant is given **TWO MONTHS** from the date of this Notice within which to file all required items and pay any fees required below to avoid abandonment. Extensions of time may be obtained by filing a petition accompanied by the extension fee under the provisions of 37 CFR 1.136(a).

- The oath or declaration is unsigned.

The application is informal since it does not comply with the regulations for the reason(s) indicated below.

The required item(s) identified below must be timely submitted to avoid abandonment:

- Replacement drawings in compliance with 37 CFR 1.84 and 37 CFR 1.121(d) are required. The drawings submitted are not acceptable because:
  - Numbers, letters, and reference characters on the drawings must measure at least 0.32 cm (1/8 inch) in height. See Figure(s) Fig 4 to 6.
  - The drawings submitted to the Office are not electronically reproducible because portions of figures Fig 1 and 3 are missing and/or blurry.

Applicant is cautioned that correction of the above items may cause the specification and drawings page count to exceed 100 pages. If the specification and drawings exceed 100 pages, applicant will need to submit the required application size fee.

The applicant needs to satisfy supplemental fees problems indicated below.

The required item(s) identified below must be timely submitted to avoid abandonment:

- To avoid abandonment, a surcharge (for late submission of filing fee, search fee, examination fee or oath or declaration) as set forth in 37 CFR 1.16(f) of $**130** for a non-small entity, must be submitted with the missing items identified in this notice.

SUMMARY OF FEES DUE:

Total additional fee(s) required for this application is $**130** for a non-small entity
- $**130** Surcharge.

Replies should be mailed to:

Mail Stop Missing Parts
Commissioner for Patents
P.O. Box 1450
Alexandria VA 22313-1450

Registered users of EFS-Web may alternatively submit their reply to this notice via EFS-Web.
https://sportal.uspto.gov/authenticate/AuthenticateUserLocalEPF.html

For more information about EFS-Web please call the USPTO Electronic Business Center at **1-866-217-9197** or visit our website at http://www.uspto.gov/ebc.

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Office of Data Management, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

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United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
12/466,213	05/14/2009	1614	5392	9516-847-999	65	4

CONFIRMATION NO. 5370

20583
JONES DAY
222 EAST 41ST ST
NEW YORK, NY 10017

FILING RECEIPT

OC000000036112150

Date Mailed: 05/28/2009

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Applicant(s)

Jeffrey B. Etter, Boulder, CO;
Mei Lai, Longmont, CO;
Jay Thomas Backstrom, Leawood, KS;

Power of Attorney: None

Domestic Priority data as claimed by applicant

This appln claims benefit of 61/053,609 05/15/2008
and claims benefit of 61/157,875 03/05/2009
and claims benefit of 61/201,145 12/05/2008

Foreign Applications

If Required, Foreign Filing License Granted: 05/21/2009

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 12/466,213**

Projected Publication Date: To Be Determined - pending completion of Missing Parts

Non-Publication Request: No

Early Publication Request: No

Title

ORAL FORMULATIONS OF CYTIDINE ANALOGS AND METHODS OF USE THEREOF

Preliminary Class

514

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4158).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as

set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign AssetsControl, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

PTO/SB/06 (12-04)

Filing Date: 05/14/09

Approved for use through 7/31/2006. OMB 0651-0032
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 displays a valid OMB control number.

PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 12/466,213

APPLICATION AS FILED – PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE ($)	SMALL ENTITY FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY FEE ($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	**330**
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	**540**
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	**220**
TOTAL CLAIMS (37 CFR 1.16(i))	**91** minus 20 =	**71**	x$26		OR	x$52	**3692**
INDEPENDENT CLAIMS (37 CFR 1.16(h))	**4** minus 3 =	* **1**	x$110			x$220	**220**
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $270 ($135 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR						
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))			195			390	**390**
			TOTAL			TOTAL	**5392**

* If the difference in column 1 is less than zero, enter "0" in column 2.

APPLICATION AS AMENDED – PART II

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE ($)	SMALL ENTITY ADDITIONAL FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE ($)
AMENDMENT A	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))					N/A		OR	N/A	
						TOTAL ADD'T FEE		OR	TOTAL ADD'T FEE	

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)	OR	RATE ($)	ADDITIONAL FEE ($)
AMENDMENT B	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))					N/A		OR	N/A	
						TOTAL ADD'T FEE		OR	TOTAL ADD'T 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.

This collection of information is required by 37 CFR 1.16. 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, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

ELECTRONIC FILING

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:	1614
Filed:	May 14, 2009	Examiner:	To Be Assigned
For:	ORAL FORMULATIONS OF CYTIDINE ANALOGS AND METHODS OF USE THEREOF	Attorney Docket No.:	9516-847-999 (501872-999847)

RESPONSE TO NOTICE TO FILE MISSING PARTS OF NON-PROVISIONAL APPLICATION FILED UNDER 37 CFR 1.53(b)

Mail Stop Missing Parts
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

In response to the Notice To File Missing Parts of Non-Provisional Application dated May 28, 2009, (hereinafter, "the Notice"), a copy of which is enclosed herewith, Applicants submit: (1) a copy of the Declaration executed by the inventors (3 counterparts); (2) replacement drawings in compliance with 37 CFR §§ 1.84 and CFR 1.121(d) (23 sheets); and (3) the required fee.

It is respectfully submitted that the replacement drawings contain no new matter. Examination is respectfully requested.

It is estimated that a fee of $130 is required for filing this Response. Please charge any required fee to Jones Day Deposit Account No. 50-3013.

Respectfully submitted,

Date: July 21, 2009

for: Anthony M. Insogna

Andrew V. Trask (Reg. No. 59,239)
for Anthony M. Insogna (Reg. No. 35,203)
JONES DAY
222 East 41st Street
New York, New York 10017-6702
(212) 326-3939

DECLARATION FOR NON-PROVISIONAL PATENT APPLICATION*

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below at 201 et seq. beneath my name.

I believe I am the original, first and sole inventor if only one name is listed at 201 below, or an original, first and joint inventor if plural names are listed at 201 et seq. below, of the subject matter which is claimed and for which a patent is sought on the invention entitled

ORAL FORMULATIONS OF CYTIDINE ANALOGS AND METHODS OF USE THEREOF

and for which a patent application:

☒ is attached hereto and includes amendment(s) filed on (if applicable)

☐ was filed in the United States on as Application No. (for declaration not accompanying application) with amendment(s) filed on (if applicable)

☐ was filed as PCT international Application No. on and was amended under PCT Article 19 on (if applicable)

I hereby authorize and request the attorneys at Jones Day to insert herein parentheses (Application No. _____ filed _____) the filing date and application number of said application when known.

I hereby state that I have reviewed and understand the contents of the above identified application, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations,§1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

EARLIEST FOREIGN APPLICATION(S), IF ANY, FILED PRIOR TO THE FILING DATE OF THE APPLICATION				
APPLICATION NUMBER	COUNTRY	DATE OF FILING (day, month, year)	PRIORITY CLAIMED	
			YES ☐	NO ☐
			YES ☐	NO ☐

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below.

PROVISIONAL APPLICATION NUMBER	FILING DATE
61/053,609	May 15, 2008
61/201,145	December 5, 2008
61/157,875	March 5, 2009

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code §112. I acknowledge the duty to disclose information known to me which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

NON-PROVISIONAL APPLICATION SERIAL NO.	FILING DATE	STATUS		
		PATENTED	PENDING	ABANDONED

* For use only when the application is assigned to a company, partnership or other organization.

I hereby declare that all statements made herein of my own knowledge are 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 the application or any patent issuing thereon.

201	FULL NAME OF INVENTOR	LAST NAME: Etter	FIRST NAME: Jeffrey	MIDDLE NAME: B.	
	RESIDENCE & CITIZENSHIP	CITY: Boulder	STATE OR FOREIGN COUNTRY: CO	COUNTRY OF CITIZENSHIP: USA	
	POST OFFICE ADDRESS	STREET: 1318 Deer Trail Road	CITY: Boulder	STATE OR COUNTRY: CO	ZIP CODE: 80302
		SIGNATURE OF INVENTOR 201: J B Etter		DATE: 18 May, 2009	
202	FULL NAME OF INVENTOR	LAST NAME: Lai	FIRST NAME: Mei	MIDDLE NAME:	
	RESIDENCE & CITIZENSHIP	CITY: Longmont	STATE OR FOREIGN COUNTRY: CO	COUNTRY OF CITIZENSHIP: USA	
	POST OFFICE ADDRESS	STREET: 1038 Neon Forest Circle	CITY: Longmont	STATE OR COUNTRY: CO	ZIP CODE: 80504
		SIGNATURE OF INVENTOR 202		DATE	
203	FULL NAME OF INVENTOR	LAST NAME: Backstrom	FIRST NAME: Jay	MIDDLE NAME: Thomas	
	RESIDENCE & CITIZENSHIP	CITY: Leawood	STATE OR FOREIGN COUNTRY: KS	COUNTRY OF CITIZENSHIP: USA	
	POST OFFICE ADDRESS	STREET: 15400 Aberdeen	CITY: Leawood	STATE OR COUNTRY: KS	ZIP CODE: 66224
		SIGNATURE OF INVENTOR 203		DATE	
204	FULL NAME OF INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME	
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP	
	POST OFFICE ADDRESS	STREET	CITY	STATE OR COUNTRY	ZIP CODE
		SIGNATURE OF INVENTOR 204		DATE	
205	FULL NAME OF INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME	
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP	
	POST OFFICE ADDRESS	STREET	CITY	STATE OR COUNTRY	ZIP CODE
		SIGNATURE OF INVENTOR 205		DATE	

DECLARATION FOR NON-PROVISIONAL PATENT APPLICATION*

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			YES ☐	NO ☐
			YES ☐	NO ☐

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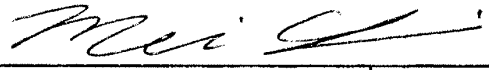
	POST OFFICE ADDRESS	STREET: 1038 Neon Forest Circle	CITY: Longmont	STATE OR COUNTRY: CO	ZIP CODE: 80504
		SIGNATURE OF INVENTOR 202 		DATE: 15 May 2009	
203	FULL NAME OF INVENTOR	LAST NAME: Backstrom	FIRST NAME: Jay	MIDDLE NAME: Thomas	
	RESIDENCE & CITIZENSHIP	CITY: Leawood	STATE OR FOREIGN COUNTRY: KS	COUNTRY OF CITIZENSHIP: USA	
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		SIGNATURE OF INVENTOR 203		DATE: 15 May 2007	
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	POST OFFICE ADDRESS	STREET	CITY	STATE OR COUNTRY	ZIP CODE
		SIGNATURE OF INVENTOR 205		DATE	

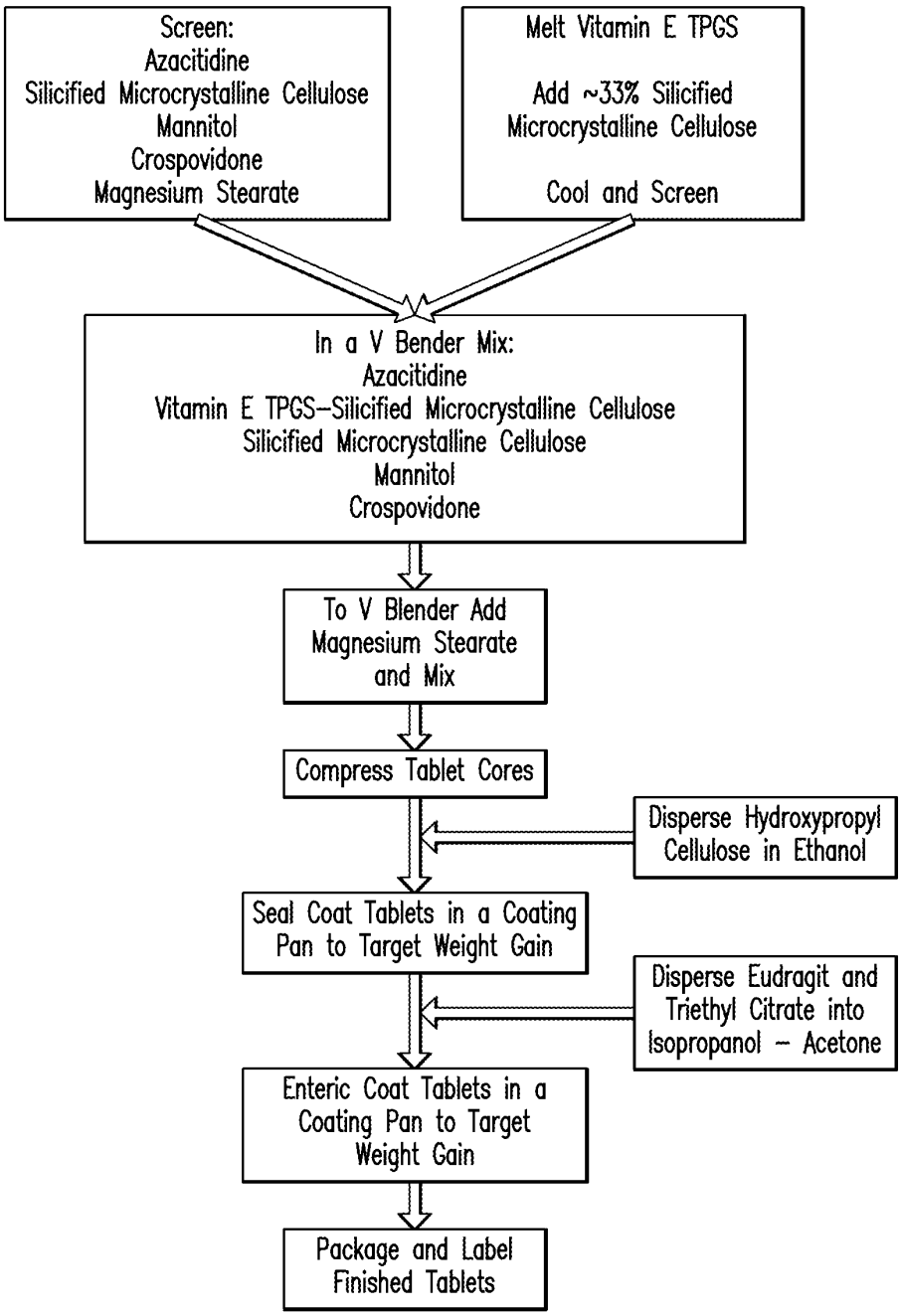
Screen:
Azacitidine
Silicified Microcrystalline Cellulose
Mannitol
Crospovidone
Magnesium Stearate
Melt Vitamin E TPGS
Add ~33% Silicified Microcrystalline Cellulose
Cool and Screen
In a V Bender Mix:
Azacitidine
Vitamin E TPGS–Silicified Microcrystalline Cellulose
Silicified Microcrystalline Cellulose
Mannitol
Crospovidone
To V Blender Add Magnesium Stearate and Mix
Compress Tablet Cores
Disperse Hydroxypropyl Cellulose in Ethanol
Seal Coat Tablets in a Coating Pan to Target Weight Gain
Disperse Eudragit and Triethyl Citrate into Isopropanol – Acetone
Enteric Coat Tablets in a Coating Pan to Target Weight Gain
Package and Label Finished Tablets

FIG. 1

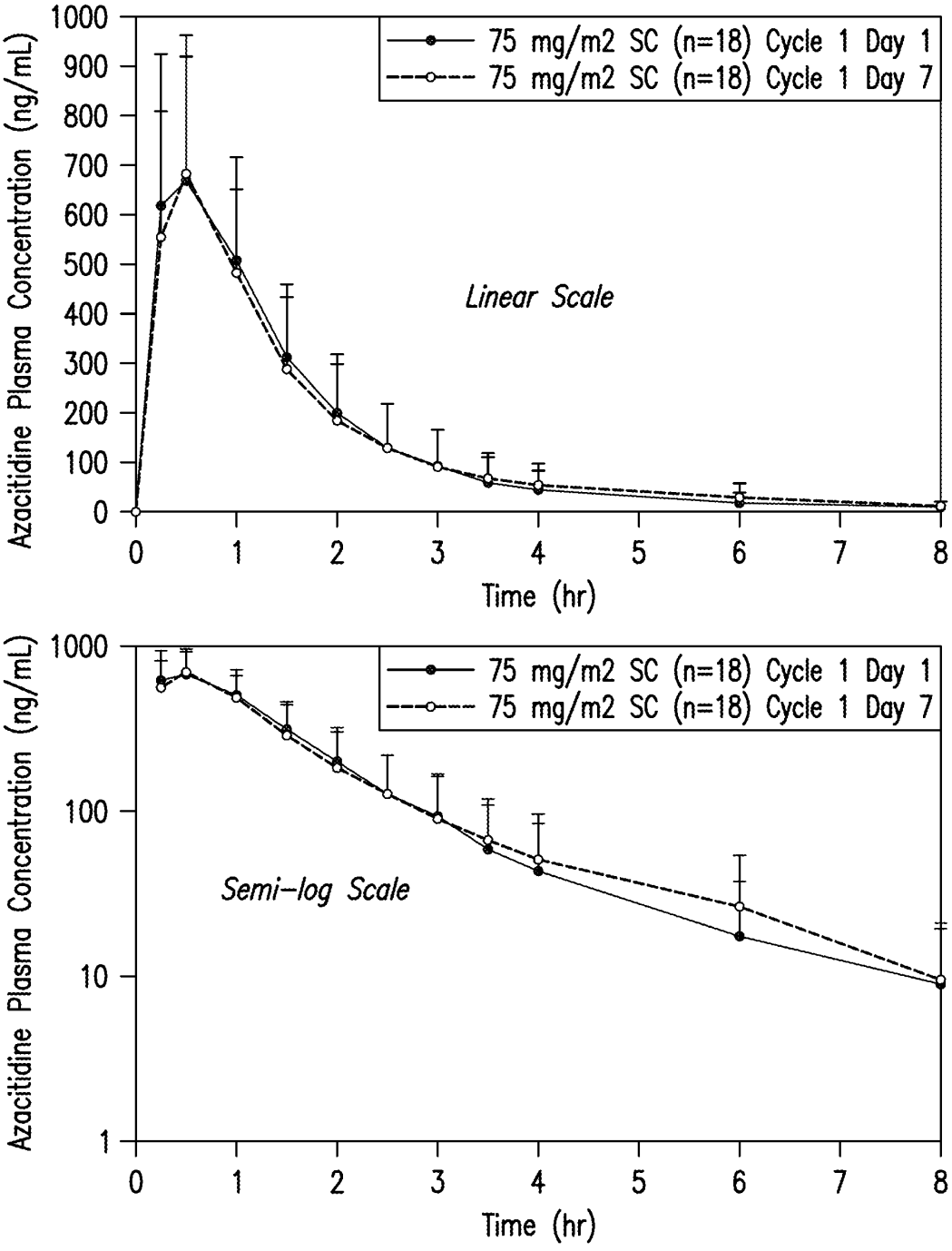
Azacitidine Plasma Concentration (ng/mL)
1000
900
800
700
600
500
400
300
200
100
0
75 mg/m2 SC (n=18) Cycle 1 Day 1
75 mg/m2 SC (n=18) Cycle 1 Day 7
Linear Scale
0
1
2
3
4
5
6
7
8
Time (hr)
Azacitidine Plasma Concentration (ng/mL)
1000
100
10
1
75 mg/m2 SC (n=18) Cycle 1 Day 1
75 mg/m2 SC (n=18) Cycle 1 Day 7
Semi-log Scale
0
1
2
3
4
5
6
7
8
Time (hr)

FIG.2

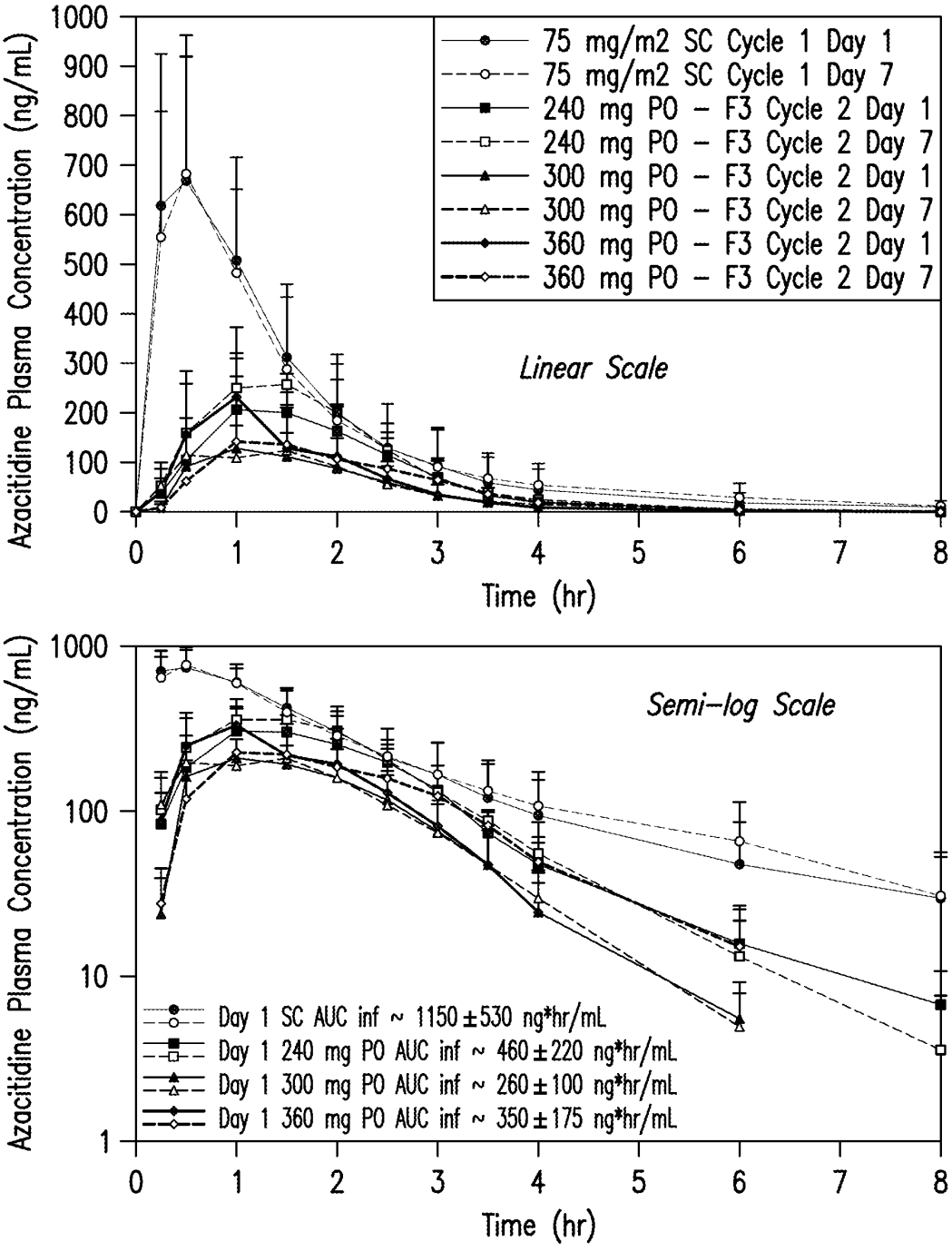
Azacitidine Plasma Concentration (ng/mL)
1000
900
800
700
600
500
400
300
200
100
0
0
1
2
3
4
5
6
7
8
Time (hr)
75 mg/m2 SC Cycle 1 Day 1
75 mg/m2 SC Cycle 1 Day 7
240 mg PO – F3 Cycle 2 Day 1
240 mg PO – F3 Cycle 2 Day 7
300 mg PO – F3 Cycle 2 Day 1
300 mg PO – F3 Cycle 2 Day 7
360 mg PO – F3 Cycle 2 Day 1
360 mg PO – F3 Cycle 2 Day 7
Linear Scale
Azacitidine Plasma Concentration (ng/mL)
1000
100
10
1
0
1
2
3
4
5
6
7
8
Time (hr)
Semi–log Scale
Day 1 SC AUC inf ~ 1150±530 ng*hr/mL
Day 1 240 mg PO AUC inf ~ 460±220 ng*hr/mL
Day 1 300 mg PO AUC inf ~ 260±100 ng*hr/mL
Day 1 360 mg PO AUC inf ~ 350±175 ng*hr/mL

FIG.3

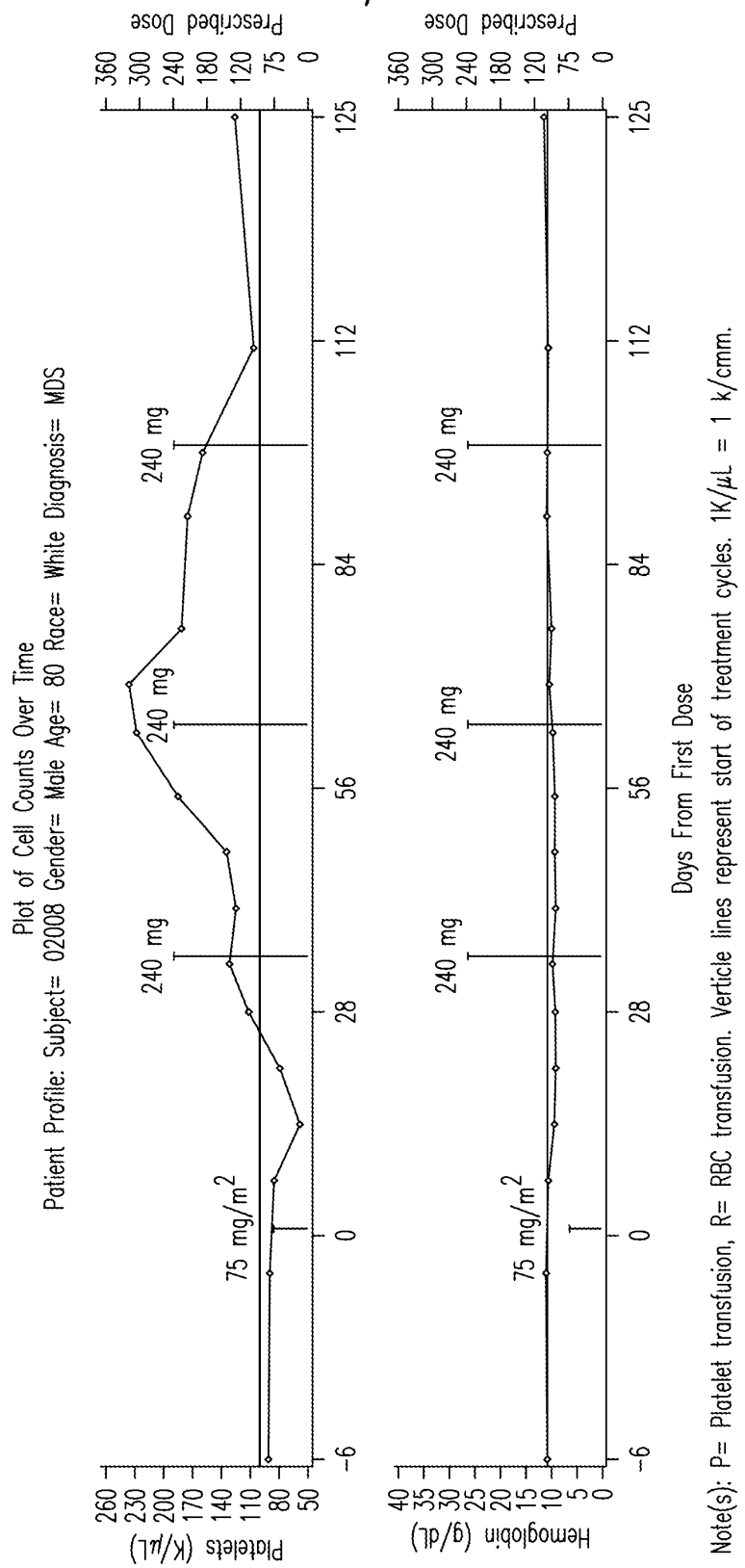
Plot of Cell Counts Over Time
Patient Profile: Subject= 02008 Gender= Male Age= 80 Race= White Diagnosis= MDS
Platelets (K/μL)
Hemoglobin (g/dL)
Prescribed Dose
75 mg/m2
240 mg
Days From First Dose
Note(s): P= Platelet transfusion, R= RBC transfusion. Verticle lines represent start of treatment cycles. 1K/μL = 1 k/cmm.

FIG.4A

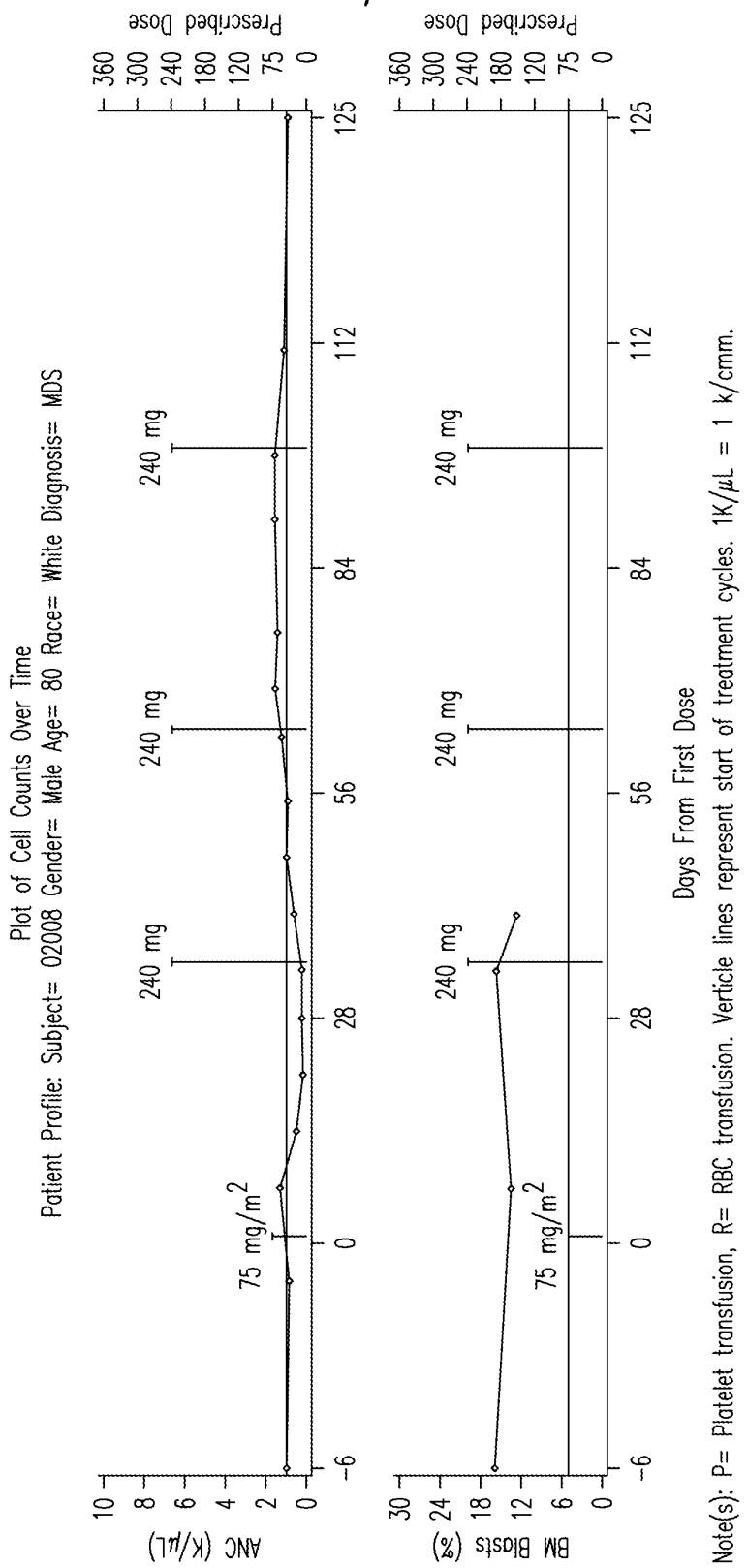
Plot of Cell Counts Over Time
Patient Profile: Subject= 02008 Gender= Male Age= 80 Race= White Diagnosis= MDS
ANC (K/μL)
BM Blasts (%)
Prescribed Dose
360
300
240
180
120
75
0
10
8
6
4
2
0
30
24
18
12
6
0
75 mg/m2
240 mg
240 mg
240 mg
−6
0
28
56
84
112
125
Days From First Dose
Note(s): P= Platelet transfusion, R= RBC transfusion. Verticle lines represent start of treatment cycles. 1K/μL = 1 k/cmm.

FIG.4B

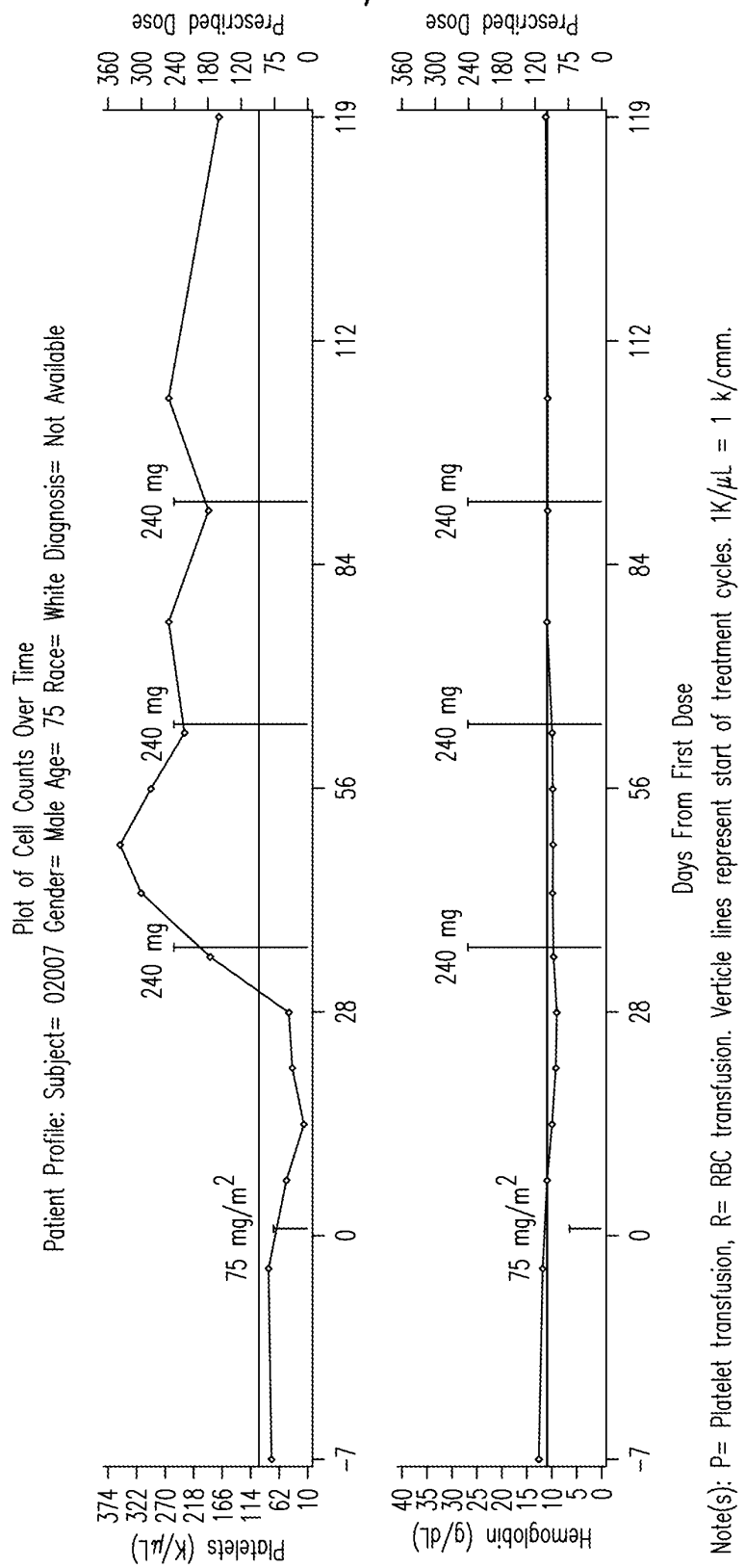
Plot of Cell Counts Over Time
Patient Profile: Subject= 02007 Gender= Male Age= 75 Race= White Diagnosis= Not Available
Platelets (K/μL)
Hemoglobin (g/dL)
Prescribed Dose
75 mg/m2
240 mg
Days From First Dose
Note(s): P= Platelet transfusion, R= RBC transfusion. Verticle lines represent start of treatment cycles. 1K/μL = 1 k/cmm.

FIG.5A

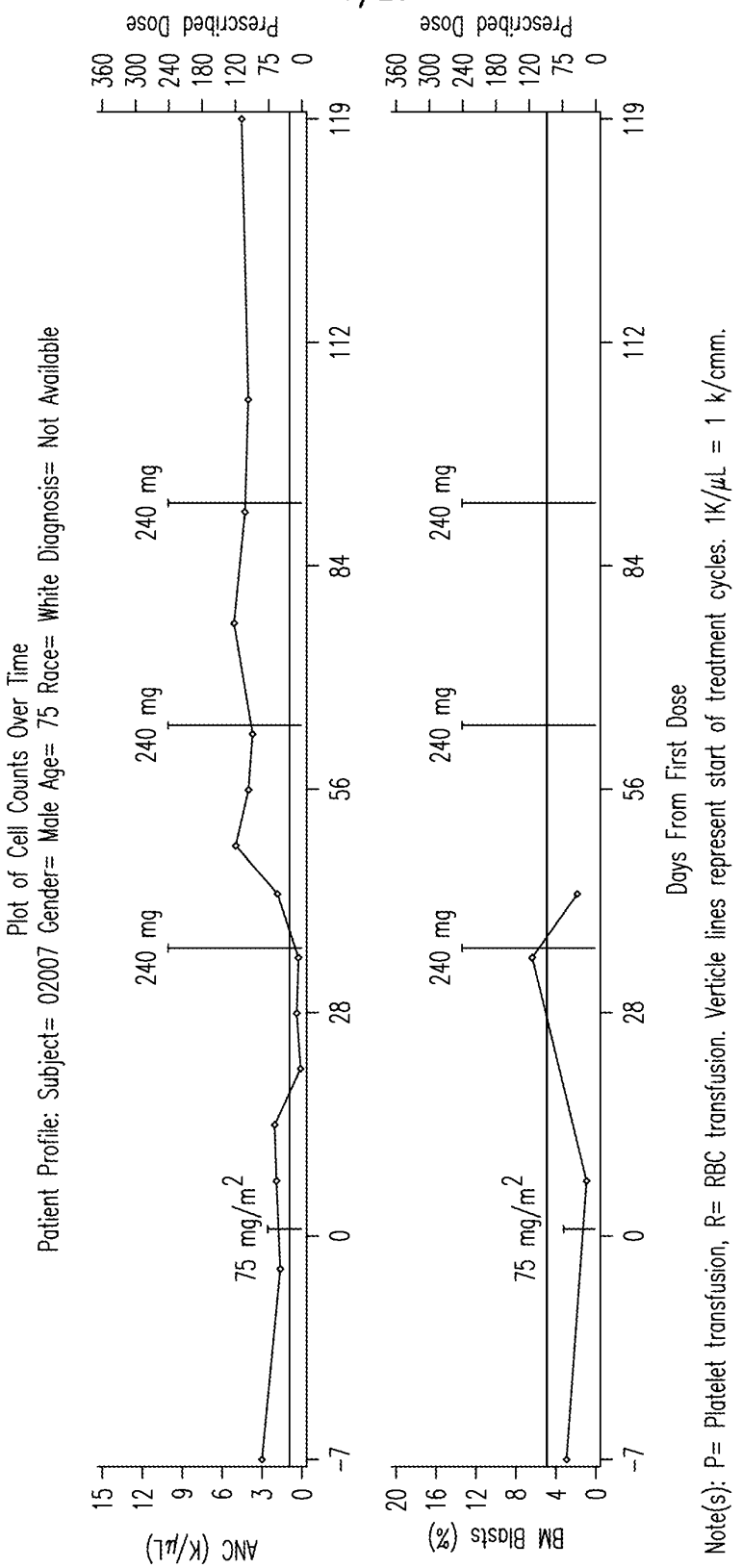
Plot of Cell Counts Over Time
Patient Profile: Subject= 02007 Gender= Male Age= 75 Race= White Diagnosis= Not Available
ANC (K/μL)
BM Blasts (%)
Prescribed Dose
360
300
240
180
120
75
0
75 mg/m2
240 mg
240 mg
240 mg
240 mg
240 mg
240 mg
15
12
9
6
3
0
20
16
12
8
4
0
-7
0
28
56
84
112
119
Days From First Dose
Note(s): P= Platelet transfusion, R= RBC transfusion. Verticle lines represent start of treatment cycles. 1K/μL = 1 k/cmm.

FIG.5B

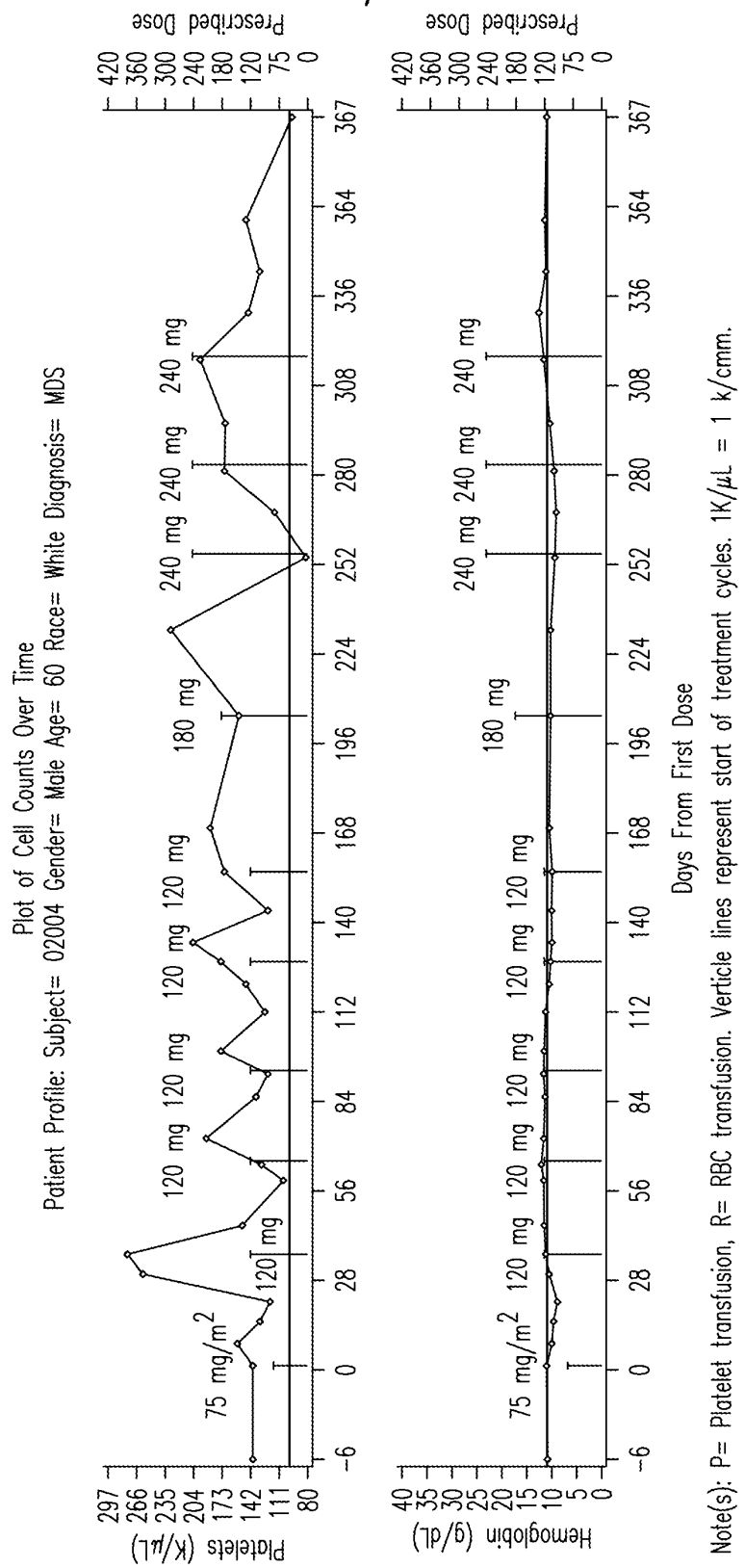
Plot of Cell Counts Over Time
Patient Profile: Subject= 02004 Gender= Male Age= 60 Race= White Diagnosis= MDS
Platelets (K/μL)
Hemoglobin (g/dL)
Prescribed Dose
75 mg/m2
120 mg
120 mg 120 mg
120 mg 120 mg 120 mg
180 mg
240 mg 240 mg
240 mg
Days From First Dose
Note(s): P= Platelet transfusion, R= RBC transfusion. Verticle lines represent start of treatment cycles. 1K/μL = 1 k/cmm.

FIG.6A

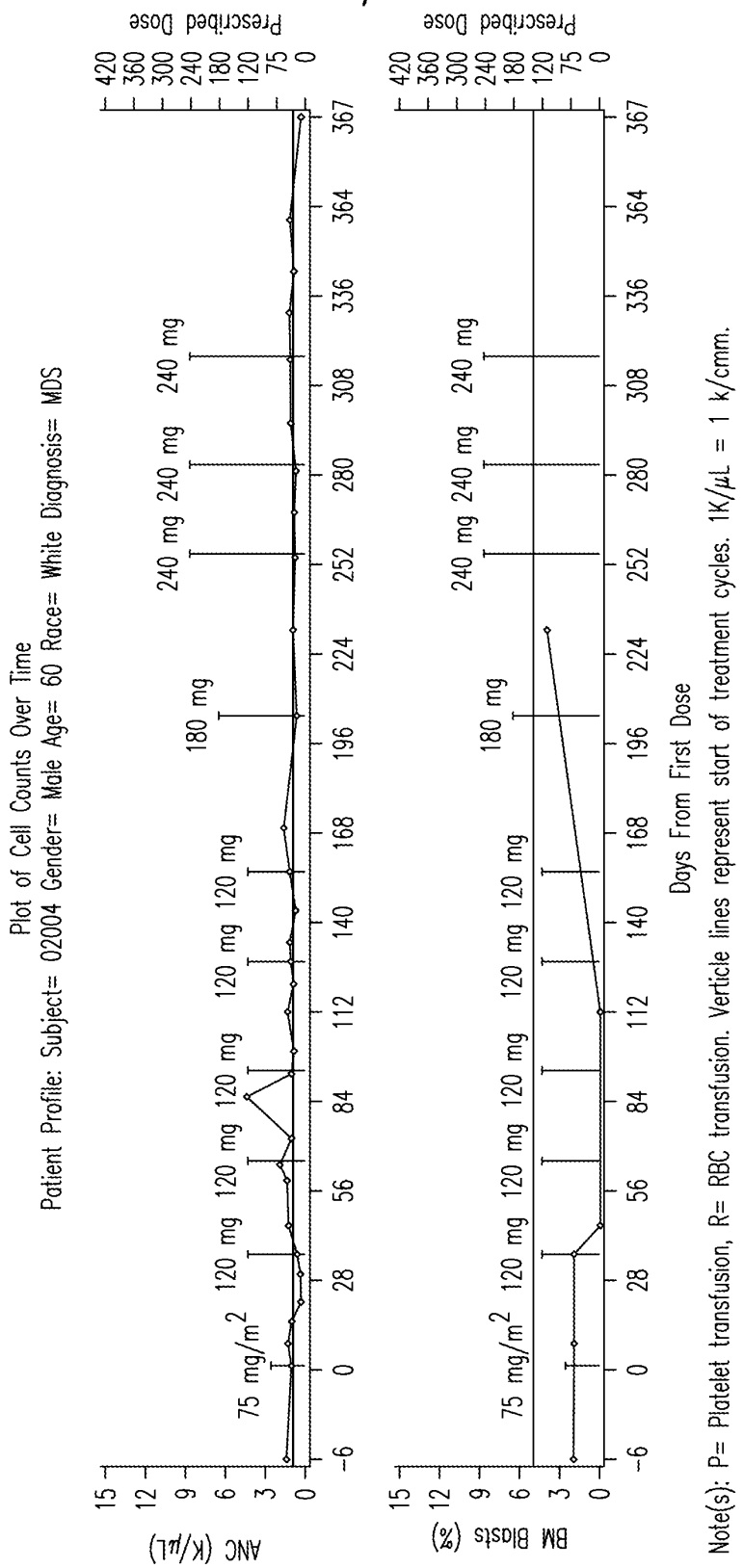
Plot of Cell Counts Over Time
Patient Profile: Subject= 02004 Gender= Male Age= 60 Race= White Diagnosis= MDS
ANC (K/μL)
BM Blasts (%)
Prescribed Dose
420
360
300
240
180
120
75
0
15
12
9
6
3
0
-6
0
28
56
84
112
140
168
196
224
252
280
308
336
364
367
75 mg/m2
120 mg
180 mg
240 mg
Days From First Dose
Note(s): P= Platelet transfusion, R= RBC transfusion. Verticle lines represent start of treatment cycles. 1K/μL = 1 k/cmm.

FIG.6B

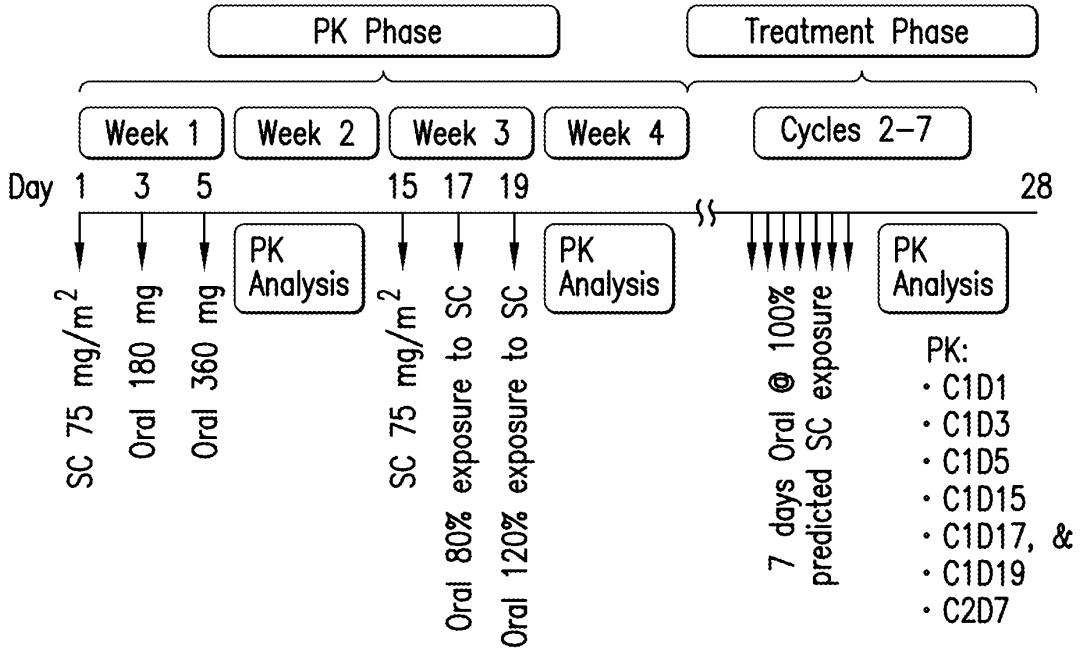
PK Phase
Treatment Phase
Week 1
Week 2
Week 3
Week 4
Cycles 2–7
Day 1 3 5 15 17 19 28
PK Analysis
PK Analysis
PK Analysis
SC 75 mg/m2
Oral 180 mg
Oral 360 mg
SC 75 mg/m2
Oral 80% exposure to SC
Oral 120% exposure to SC
7 days Oral @ 100% predicted SC exposure
PK:
• C1D1
• C1D3
• C1D5
• C1D15
• C1D17, &
• C1D19
• C2D7

FIG. 7

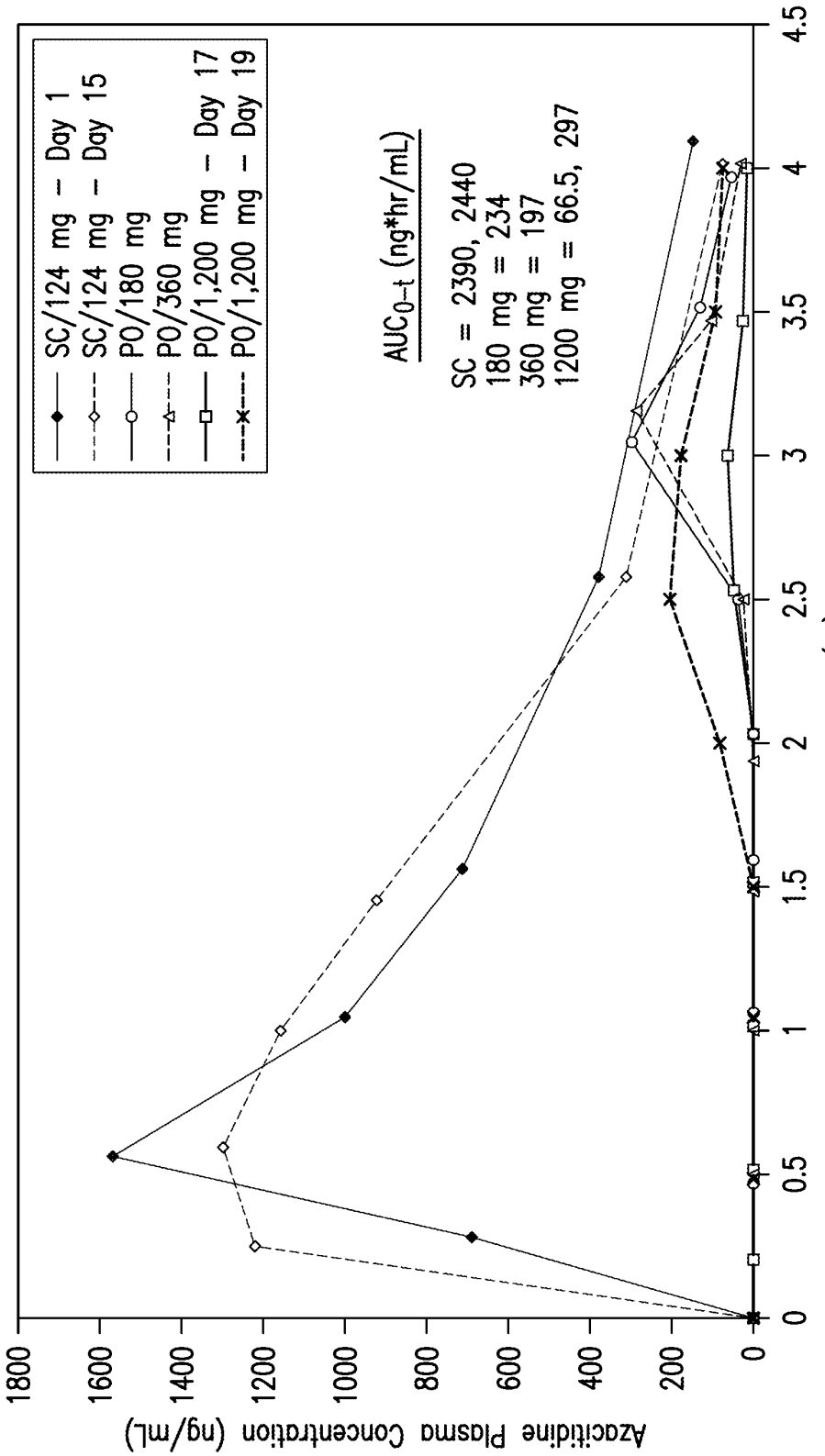
SC/124 mg – Day 1
SC/124 mg – Day 15
PO/180 mg
PO/360 mg
PO/1,200 mg – Day 17
PO/1,200 mg – Day 19
AUC0–t (ng*hr/mL)
SC = 2390, 2440
180 mg = 234
360 mg = 197
1200 mg = 66.5, 297
Azacitidine Plasma Concentration (ng/mL)
Time (hr)
0
200
400
600
800
1000
1200
1400
1600
1800
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5

FIG.8

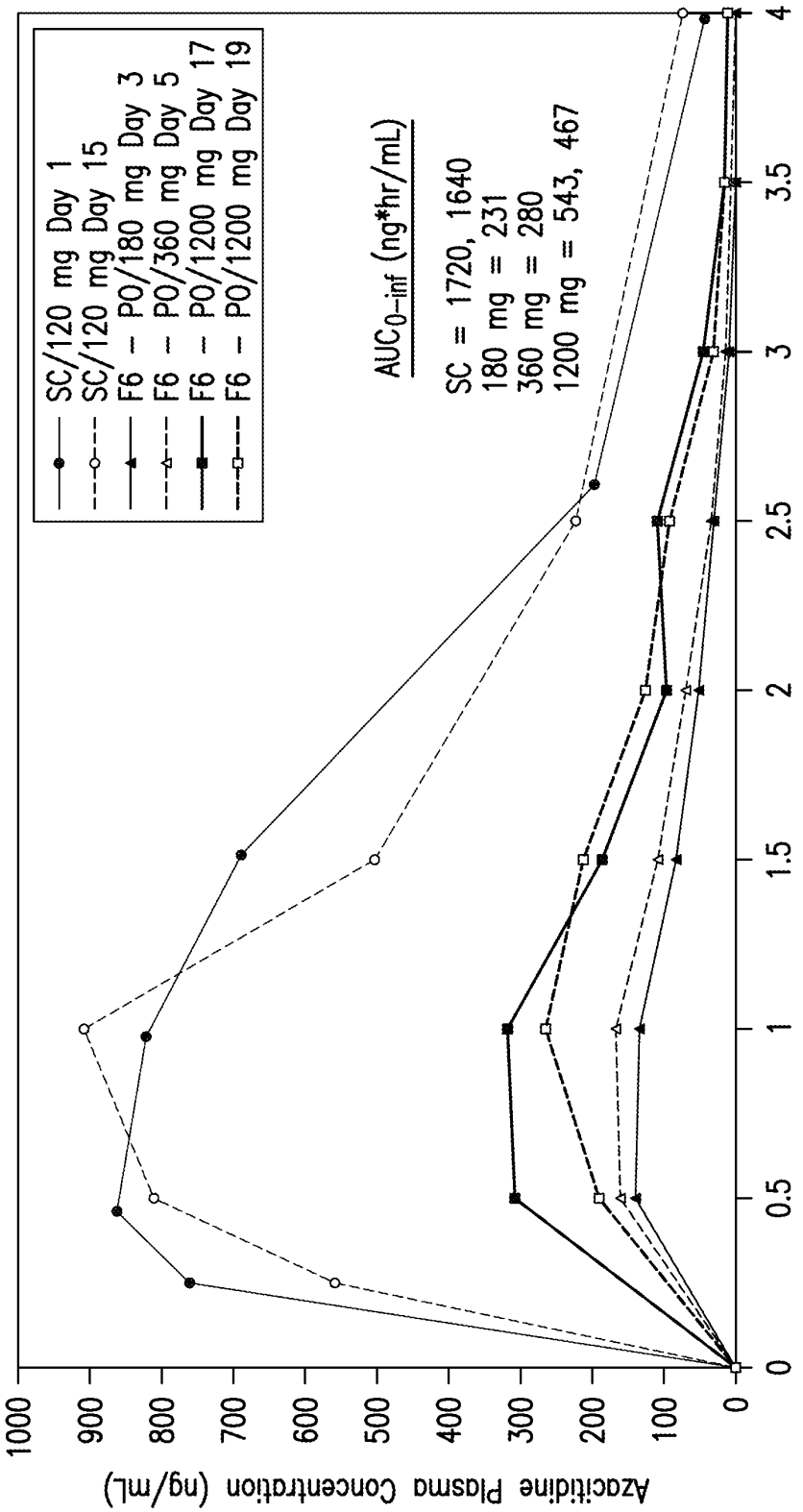
SC/120 mg Day 1
SC/120 mg Day 15
F6 -- PO/180 mg Day 3
F6 -- PO/360 mg Day 5
F6 -- PO/1200 mg Day 17
F6 -- PO/1200 mg Day 19
AUC0-inf (ng*hr/mL)
SC = 1720, 1640
180 mg = 231
360 mg = 280
1200 mg = 543, 467
Azacitidine Plasma Concentration (ng/mL)
Time (hr)
0
100
200
300
400
500
600
700
800
900
1000
0
0.5
1
1.5
2
2.5
3
3.5
4

FIG.9

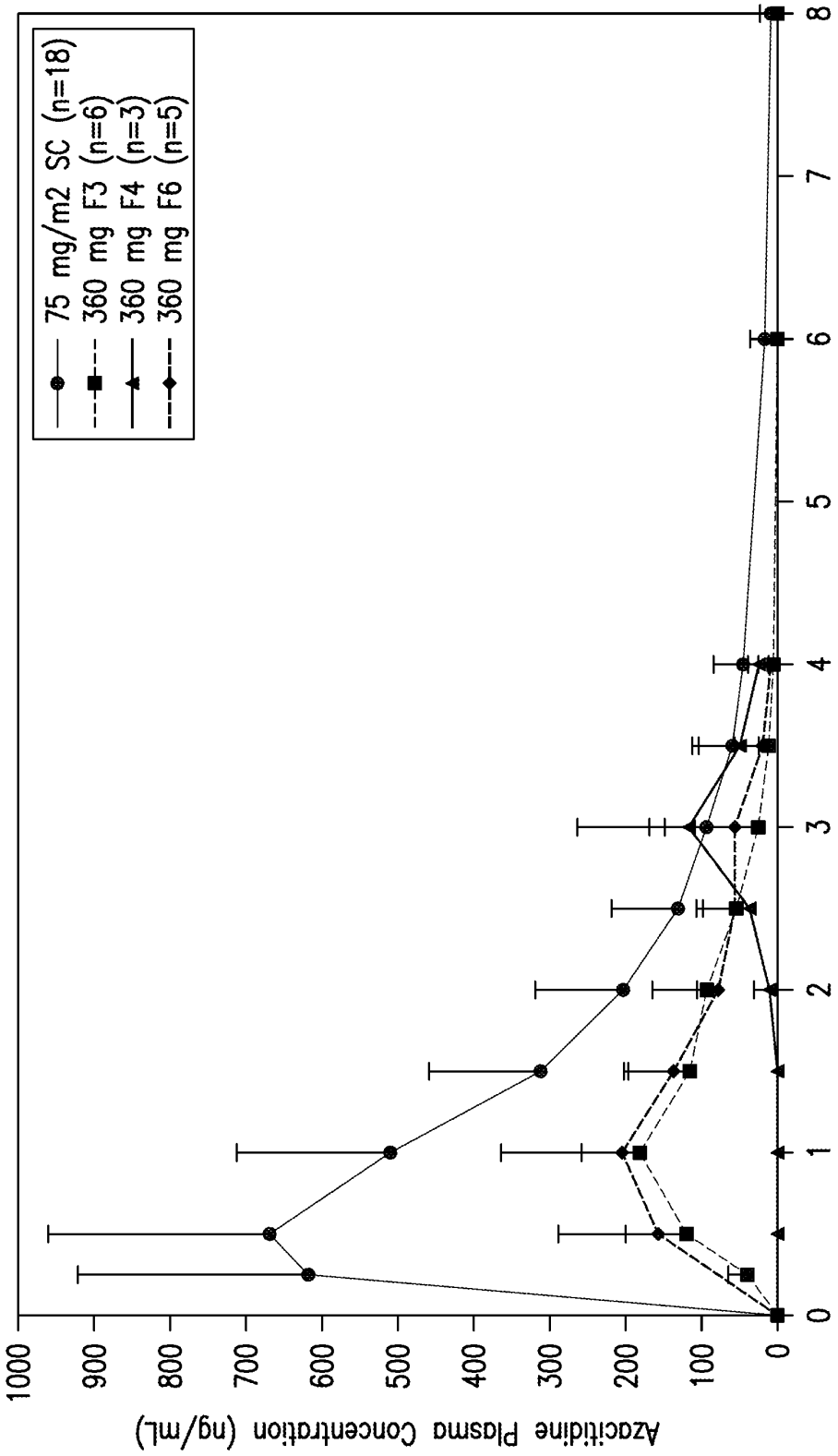
75 mg/m2 SC (n=18)
360 mg F3 (n=6)
360 mg F4 (n=3)
360 mg F6 (n=5)
Azacitidine Plasma Concentration (ng/mL)
1000
900
800
700
600
500
400
300
200
100
0
0
1
2
3
4
5
6
7
8
Time (hr)

FIG.1O

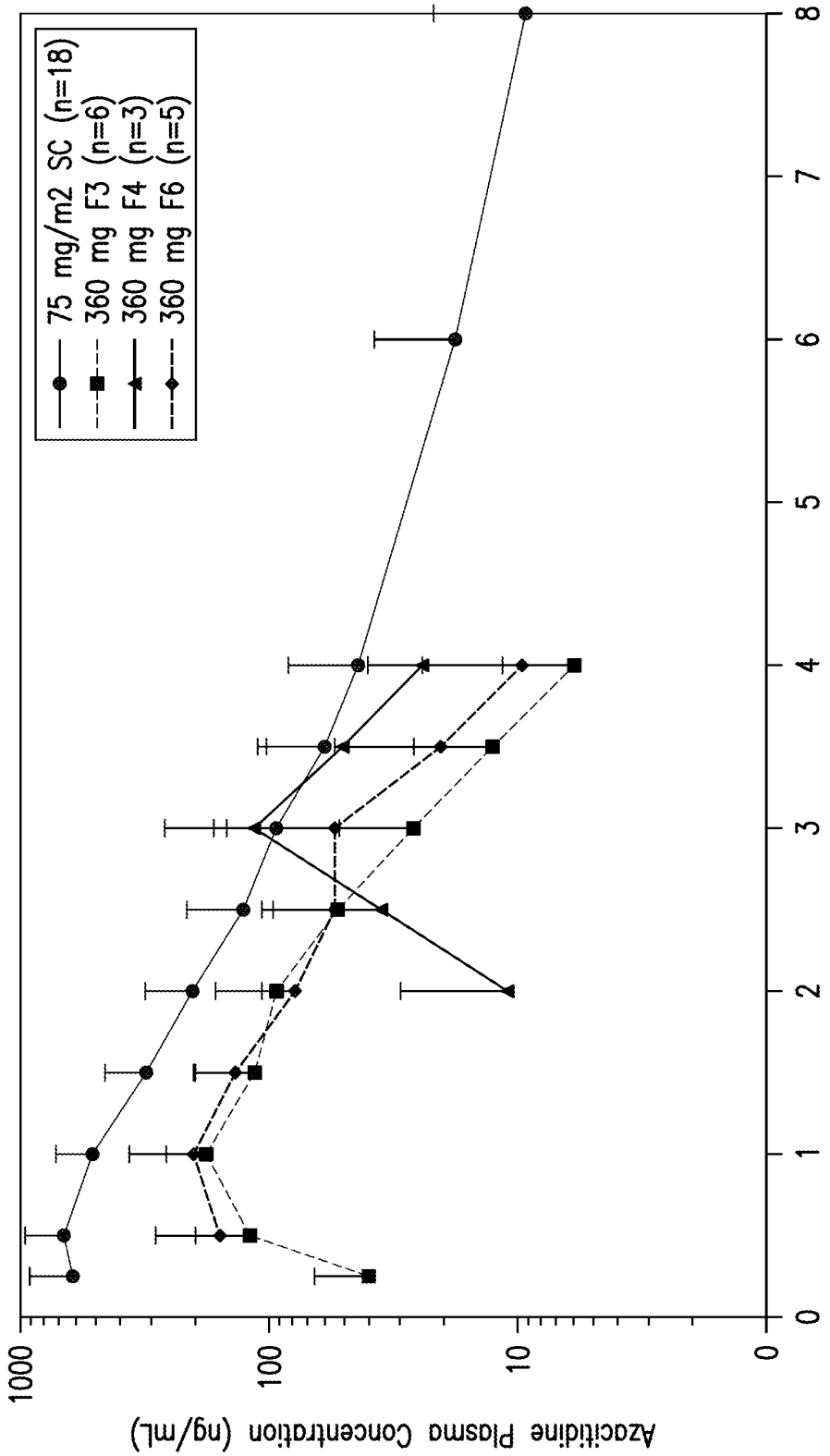
75 mg/m2 SC (n=18)
360 mg F3 (n=6)
360 mg F4 (n=3)
360 mg F6 (n=5)
Azacitidine Plasma Concentration (ng/mL)
1000
100
10
0
Time (hr)
0
1
2
3
4
5
6
7
8

FIG. 11

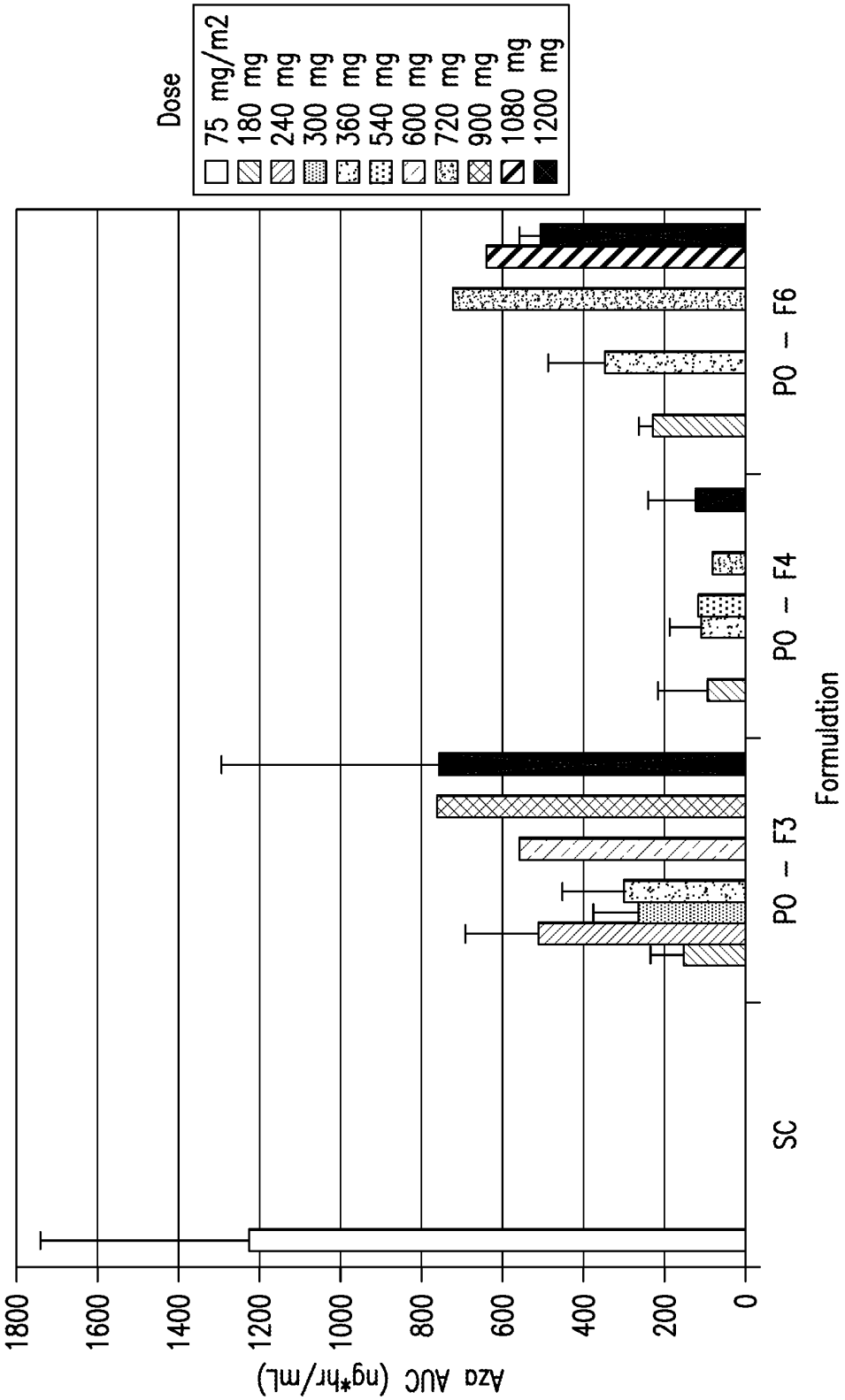
Dose
75 mg/m2
180 mg
240 mg
300 mg
360 mg
540 mg
600 mg
720 mg
900 mg
1080 mg
1200 mg
Aza AUC (ng*hr/mL)
1800
1600
1400
1200
1000
800
600
400
200
0
SC
PO – F3
PO – F4
PO – F6
Formulation

FIG. 12

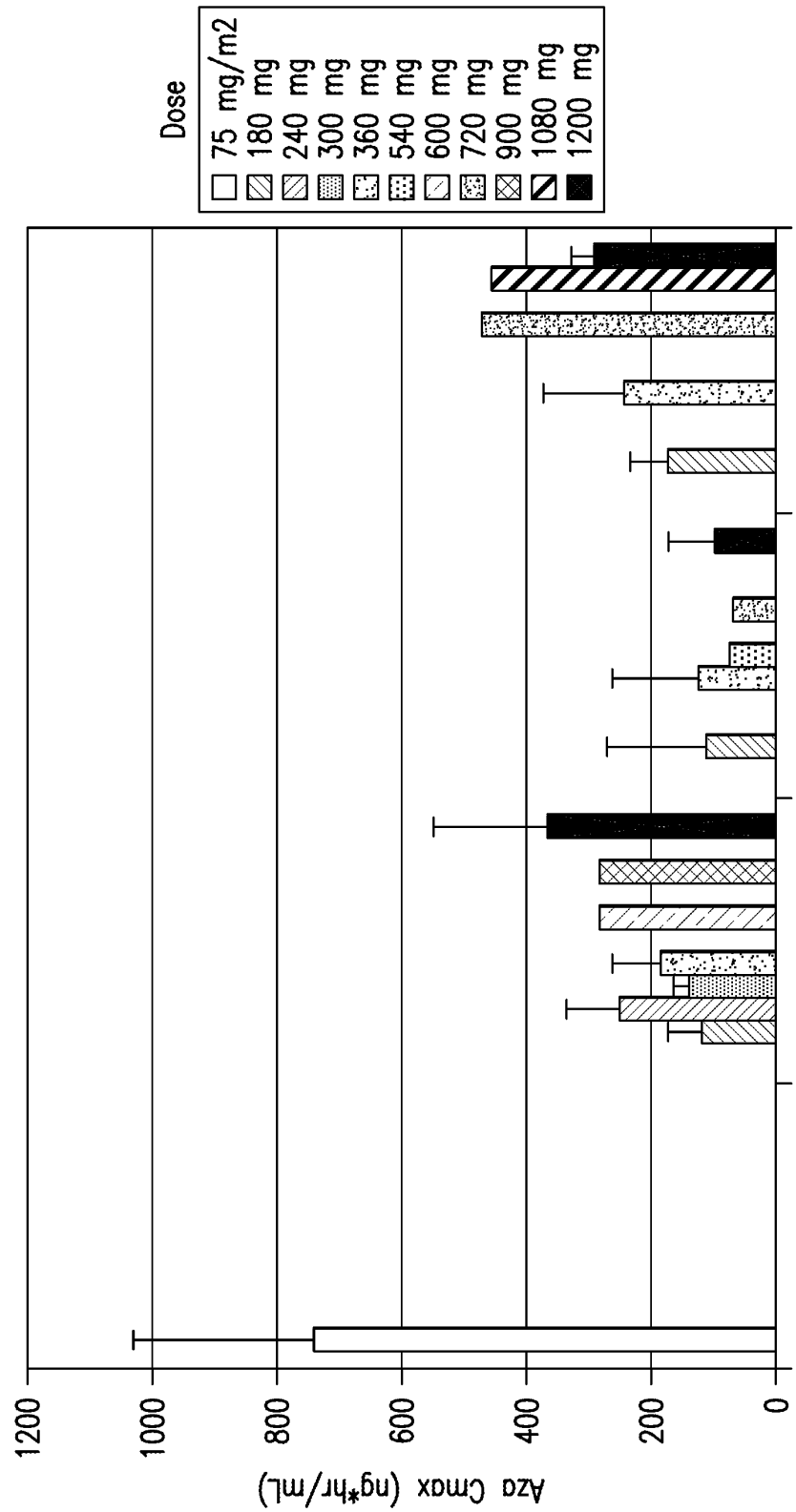
Dose
75 mg/m2
180 mg
240 mg
300 mg
360 mg
540 mg
600 mg
720 mg
900 mg
1080 mg
1200 mg
Aza Cmax (ng*hr/mL)
0
200
400
600
800
1000
1200
SC
PO – F3
PO – F4
PO – F6
Formulation

FIG.13

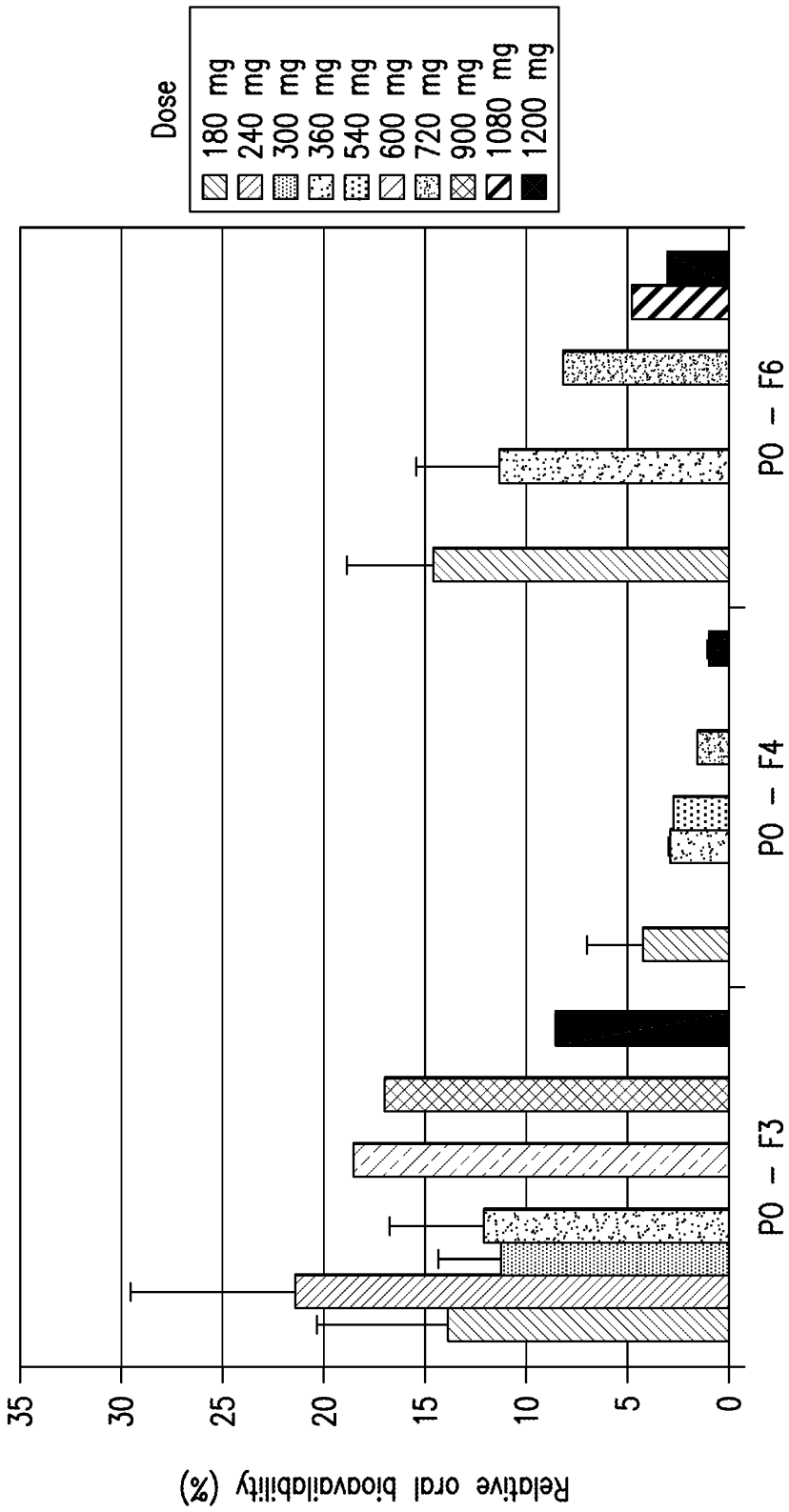
Dose
180 mg
240 mg
300 mg
360 mg
540 mg
600 mg
720 mg
900 mg
1080 mg
1200 mg
Relative oral bioavailability (%)
35
30
25
20
15
10
5
0
PO – F3
PO – F4
PO – F6
Formulation

FIG.14

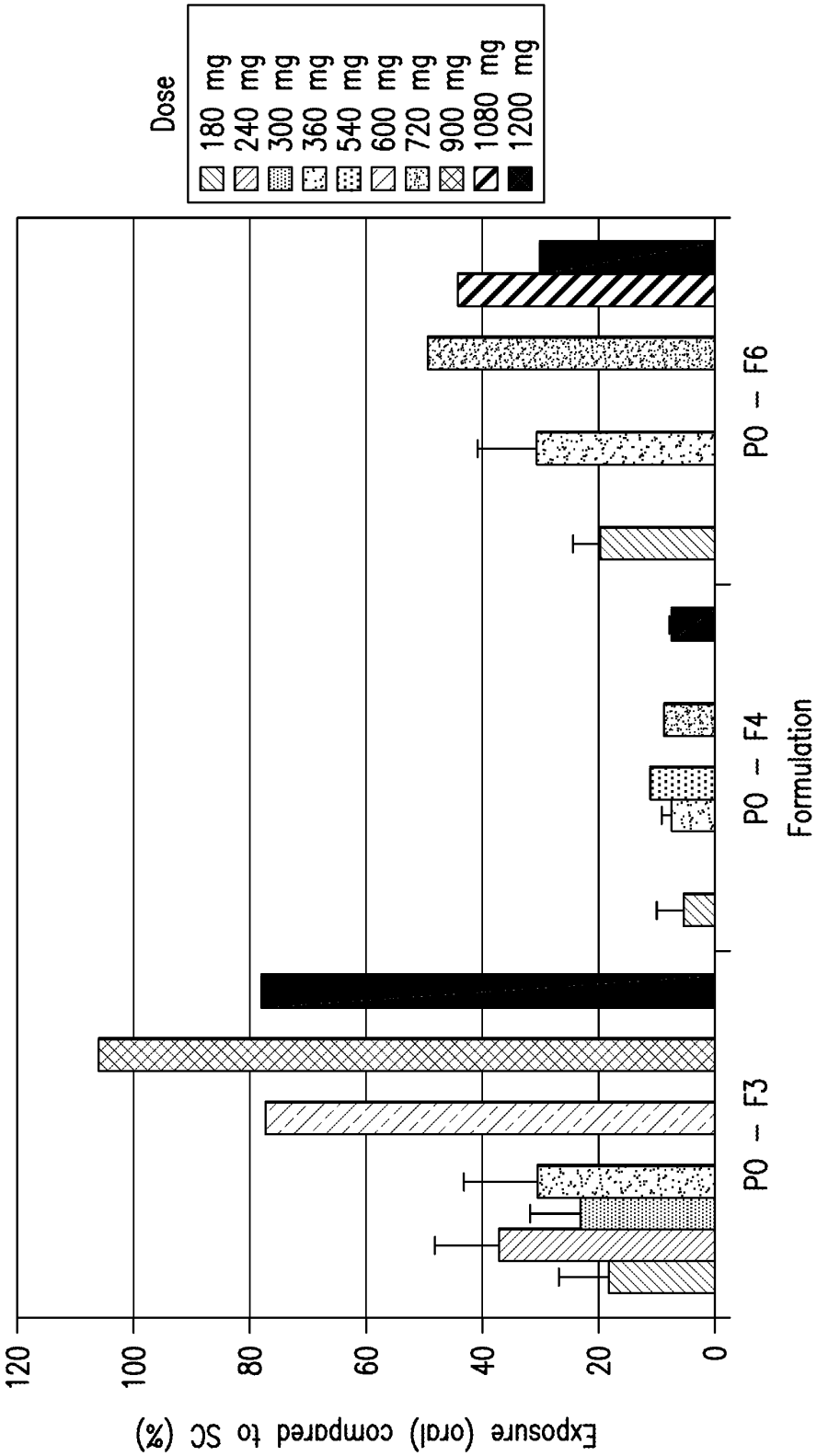
Dose
180 mg
240 mg
300 mg
360 mg
540 mg
600 mg
720 mg
900 mg
1080 mg
1200 mg
Exposure (oral) compared to SC (%)
120
100
80
60
40
20
0
P0 – F3
P0 – F4
P0 – F6
Formulation

FIG. 15

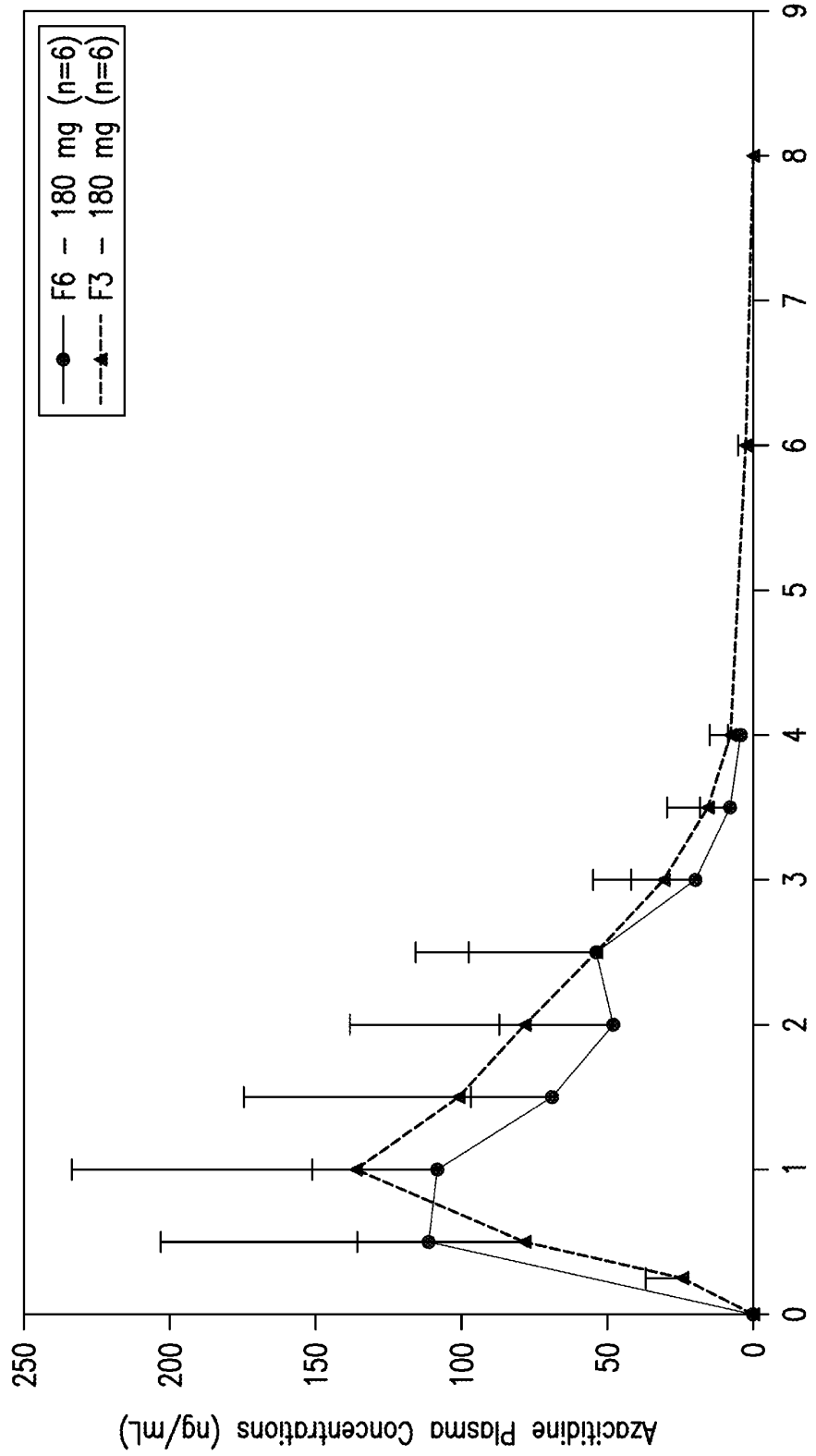
F6 – 180 mg (n=6)
F3 – 180 mg (n=6)
Azacitidine Plasma Concentrations (ng/mL)
250
200
150
100
50
0
0
1
2
3
4
5
6
7
8
9
Time (hr)

FIG.16

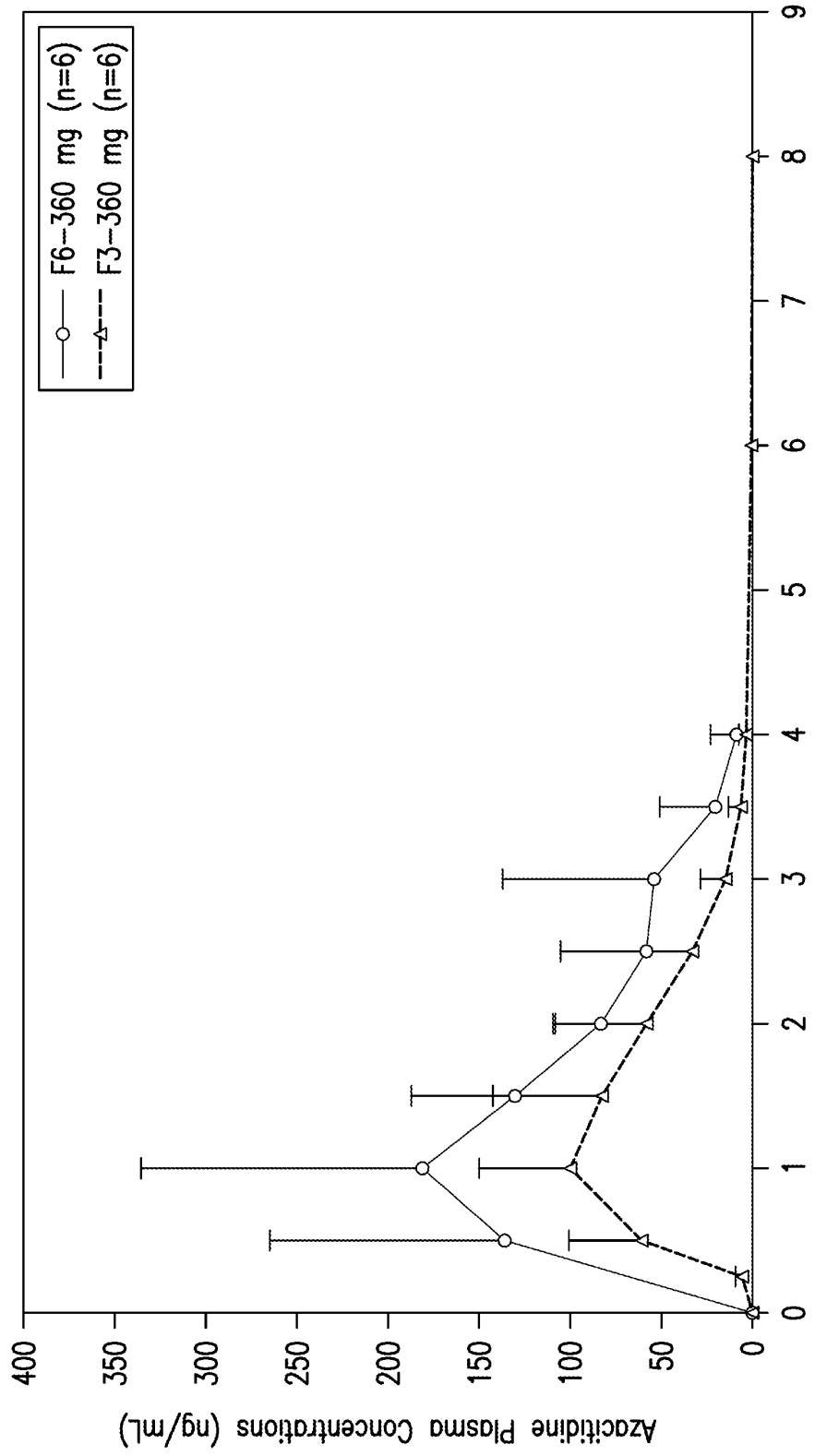
F6-360 mg (n=6)
F3-360 mg (n=6)
Azacitidine Plasma Concentrations (ng/mL)
Time (hr)
0
50
100
150
200
250
300
350
400
0
1
2
3
4
5
6
7
8
9

FIG.17

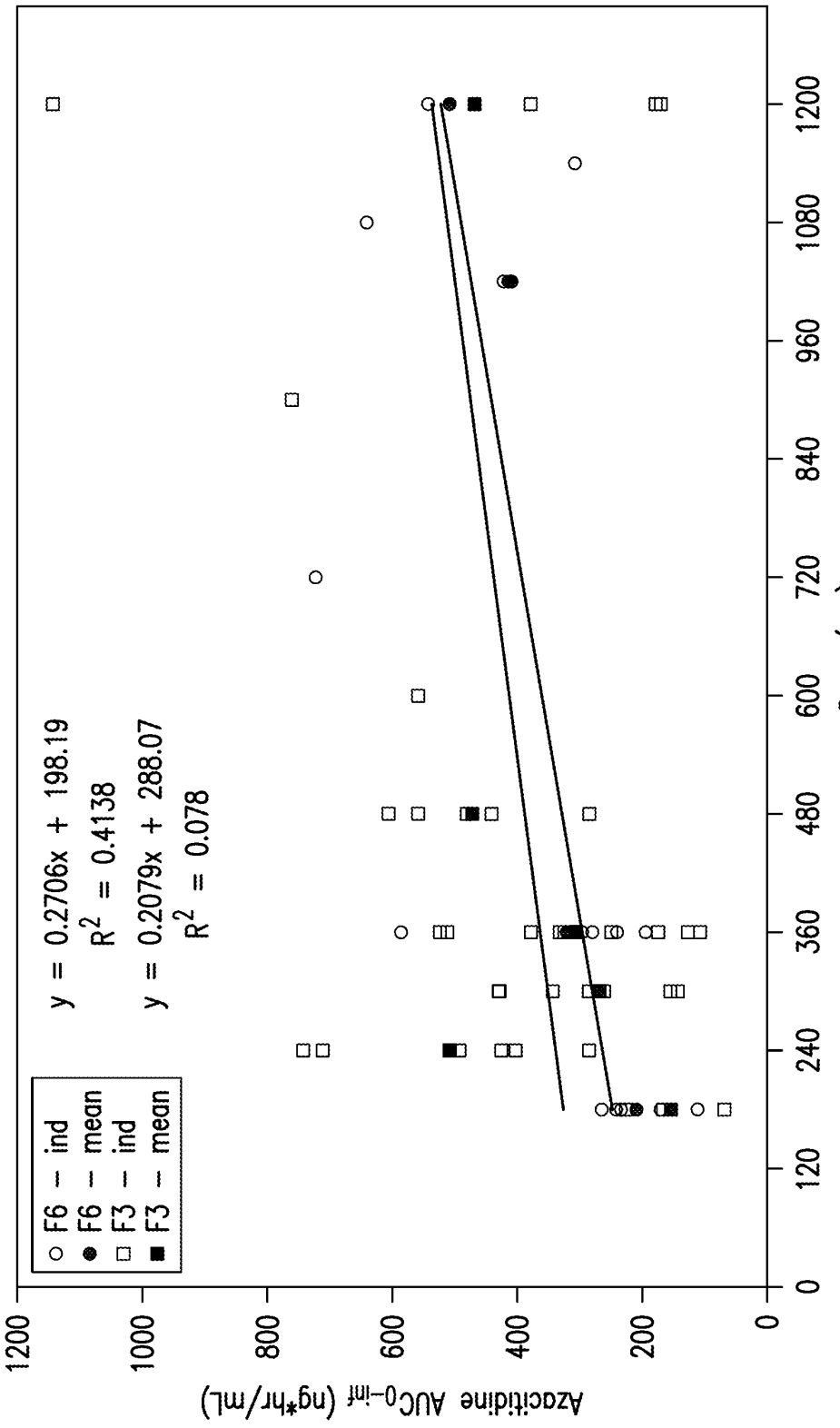
Azacitidine AUC0–inf (ng*hr/mL)
Dose (mg)
y = 0.2706x + 198.19
R2 = 0.4138
y = 0.2079x + 288.07
R2 = 0.078
F6 – ind
F6 – mean
F3 – ind
F3 – mean
0
200
400
600
800
1000
1200
120
240
360
480
600
720
840
960
1080
1200

FIG.18

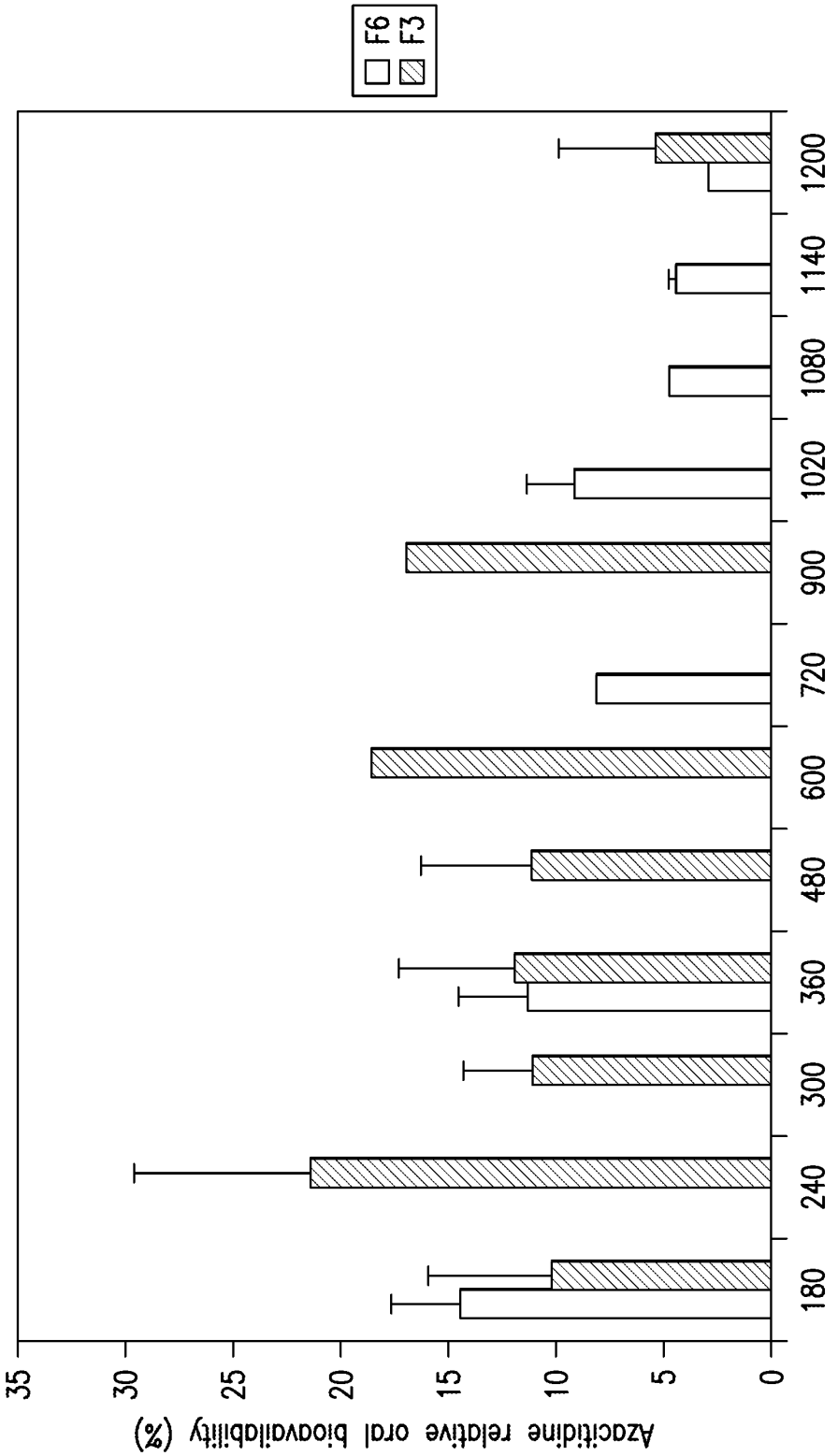
F6
F3
Azacitidine relative oral bioavailability (%)
0
5
10
15
20
25
30
35
180
240
300
360
480
600
720
900
1020
1080
1140
1200
Dose (mg)

FIG. 19

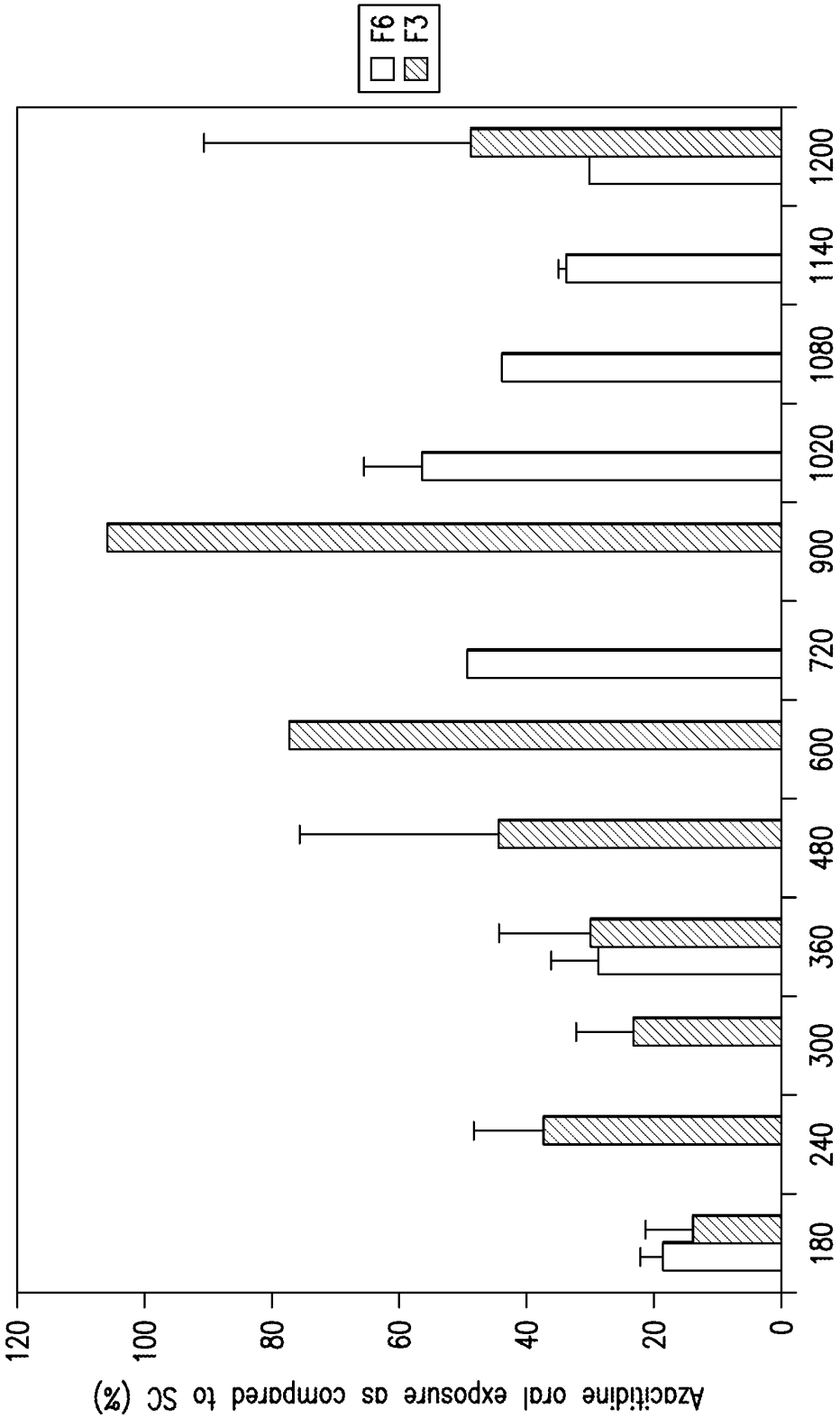
F6
F3
Azacitidine oral exposure as compared to SC (%)
0
20
40
60
80
100
120
180
240
300
360
480
600
720
900
1020
1080
1140
1200
Dose (mg)

FIG.2O

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:	Anthony M. Insogna/Rochelle Flowers
Attorney Docket Number:	9516-847-999

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:				
Late filing fee for oath or declaration	1051	1	130	130
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
			Total in USD ($)	**130**

Electronic Acknowledgement Receipt

EFS ID:	5741733
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:	20583
Filer:	Anthony M. Insogna/Rochelle Flowers
Filer Authorized By:	Anthony M. Insogna
Attorney Docket Number:	9516-847-999
Receipt Date:	21-JUL-2009
Filing Date:	14-MAY-2009
Time Stamp:	15:42:05
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$130
RAM confirmation Number	1781
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	Applicant Response to Pre-Exam Formalities Notice	Response_to_Notice_to_File_Missing_Parts.pdf	31844 17aa7a4ddbcd2c354bbfd50e41028f11fda28d60	no	1
Warnings:					
Information:					
2	Oath or Declaration filed	Declaration.pdf	312074 49681a2b2df08a10c15b03f3889fcbd7e9ee0f97	no	6
Warnings:					
Information:					
3	Drawings-only black and white line drawings	Replacement_drawings.pdf	2353300 75947ef6a58849a2890150f90331650b21114fe6	no	23
Warnings:					
Information:					
4	Fee Worksheet (PTO-875)	fee-info.pdf	30110 f511b2ff795d53c9fbb992ee84ad13ad519490a3	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	2727328		

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<u>New Applications Under 35 U.S.C. 111</u>
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.

<u>National Stage of an International Application under 35 U.S.C. 371</u>
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.

<u>New International Application Filed with the USPTO as a Receiving Office</u>
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.

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UNITED STATES DEPARTMENT OF COMMERCE
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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
12/466,213	05/14/2009	1614	5522	9516-847-999	65	4

CONFIRMATION NO. 5370

20583
JONES DAY
222 EAST 41ST ST
NEW YORK, NY 10017

UPDATED FILING RECEIPT

OC000000037079835

Date Mailed: 07/28/2009

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Applicant(s)

Jeffrey B. Etter, Boulder, CO;
Mei Lai, Longmont, CO;
Jay Thomas Backstrom, Leawood, KS;

Power of Attorney: None

Domestic Priority data as claimed by applicant

This appln claims benefit of 61/053,609 05/15/2008
and claims benefit of 61/157,875 03/05/2009
and claims benefit of 61/201,145 12/05/2008

Foreign Applications

If Required, Foreign Filing License Granted: 05/21/2009

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 12/466,213**

Projected Publication Date: 11/19/2009

Non-Publication Request: No

Early Publication Request: No

Title

ORAL FORMULATIONS OF CYTIDINE ANALOGS AND METHODS OF USE THEREOF

Preliminary Class

514

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4158).

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NOT GRANTED

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DocCode - SCORE

SCORE Placeholder Sheet for IFW Content

Application Number: 12466213 Document Date: 7/21/2009

The presence of this form in the IFW record indicates that the following document type was received in electronic format on the date identified above. This content is stored in the SCORE database.

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Since this was an electronic submission, there is no physical artifact folder, no artifact folder is recorded in PALM, and no paper documents or physical media exist. The TIFF images in the IFW record were created from the original documents that are stored in SCORE.

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- Examiners may access SCORE content via the eDAN interface.
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Form Revision Date: February 8, 2006

ELECTRONIC FILING

PTO/SB/96 (09-04)
Approved for use through 07/31/2006. 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 displays a valid OMB control number.

STATEMENT UNDER 37 CFR 3.73(b)

Applicant/Patent Owner:	Celgene Corporation		
Application No./Patent No.:	12/466,213	Filed/Issue Date:	May 14, 2009
Entitled:	ORAL FORMULATIONS OF CYTIDINE ANALOGS AND METHODS OF USE THEREOF		
Celgene Corporation (Name of Assignee)	,	a	corporation (Type of Assignee, e.g., corporation, partnership, university, government agency, etc.)

states that it is:

1. ☒ the assignee of the entire right, title, and interest; or
2. ☐ an assignee of less than the entire right, title and interest. The extent (by percentage) of its ownership interest is %

in the patent application/patent identified above by virtue of either:

A. ☒ An assignment from the inventor(s) of the patent application/patent identified above. The assignment was recorded in the United States Patent and Trademark Office at Reel 022983, Frame 0961, or for which a copy thereof is attached.

OR

B. ☐ A chain of title from the inventor(s), of the patent application/patent identified above, to the current assignee as shown below:

1. From: To: The document was recorded in the United States Patent and Trademark Office at Reel , Frame , or for which a copy thereof is attached.
2. From: To: The document was recorded in the United States Patent and Trademark Office at Reel , Frame or for which a copy thereof is attached.
3. From: To: The document was recorded in the United States Patent and Trademark Office at Reel , Frame , or for which a copy thereof is attached.

☐ Additional documents in the chain of title are listed on a supplemental sheet.

☐ Copies of assignments or other documents in the chain of title are attached.

[NOTE: A separate copy (*i.e.*, the original assignment document or a true copy of the original document) must be submitted to Assignment Division in accordance with 37 CFR Part 3, if the assignment is to be recorded in the records of the USPTO. See MPEP 02.08]

The undersigned (whose title is supplied below) is authorized to act on behalf of the assignee.

for: Anthony M. Insogna	05 August 2009
Signature	**Date**
Andrew V. Trask	212-326-3939
for Anthony M. Insogna Reg. No. 35,203	**Telephone Number**
Printed or Typed name	
Title: Agent Reg. No. 59,239	

This collection of information is required by 37 CFR 3.73(b). 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, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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NYI- 4205761v1

PTO/SB/80 (11-08)
Approved for use through 11/30/2011. OMB 0651-0035
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

POWER OF ATTORNEY TO PROSECUTE APPLICATIONS BEFORE THE USPTO

I hereby revoke all previous powers of attorney given in the application identified in the attached statement under 37 CFR 3.73(b).

I hereby appoint:

☑ Practitioners associated with the Customer Number: 84802

OR

☐ Practitioner(s) named below (if more than ten patent practitioners are to be named, then a customer number must be used):

Name	Registration Number	Name	Registration Number

as attorney(s) or agent(s) to represent the undersigned before the United States Patent and Trademark Office (USPTO) in connection with any and all patent applications assigned only to the undersigned according to the USPTO assignment records or assignment documents attached to this form in accordance with 37 CFR 3.73(b).

Please change the correspondence address for the application identified in the attached statement under 37 CFR 3.73(b) to:

☐ The address associated with Customer Number:

OR

☐ Firm or Individual Name			
Address			
City		State	Zip
Country			
Telephone		Email	

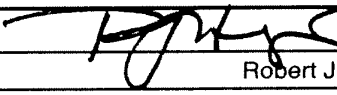
Assignee Name and Address:

Celgene Corporation
86 Morris Avenue
Summit, New Jersey 07901

A copy of this form, together with a statement under 37 CFR 3.73(b) (Form PTO/SB/96 or equivalent) is required to be filed in each application in which this form is used. The statement under 37 CFR 3.73(b) may be completed by one of the practitioners appointed in this form if the appointed practitioner is authorized to act on behalf of the assignee, and must identify the application in which this Power of Attorney is to be filed.

SIGNATURE of Assignee of Record

The individual whose signature and title is supplied below is authorized to act on behalf of the assignee

Signature		Date	March 24 2006
Name	Robert J. Hugin	Telephone	908-673-9000
Title	Chief Operating Officer and President		

This collection of information is required by 37 CFR 1.31, 1.32 and 1.33. 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 1.11 and 1.14. This collection is estimated to take 3 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, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Electronic Acknowledgement Receipt

EFS ID:	5834490
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:	20583
Filer:	Anthony M. Insogna/Rochelle Flowers
Filer Authorized By:	Anthony M. Insogna
Attorney Docket Number:	9516-847-999
Receipt Date:	05-AUG-2009
Filing Date:	14-MAY-2009
Time Stamp:	16:01:47
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	Power of Attorney	POA_w_373b_statement.pdf	135337 d81feb8a3e20d7d56c192be1c3a516167cfefedb	no	2

Warnings:

Information:

Total Files Size (in bytes):	135337

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.

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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
12/466,213	05/14/2009	Jeffrey B. Etter	9516-847-999

CONFIRMATION NO. 5370

84802
JONES DAY
222 E. 41ST. STREET
NEW YORK, NY 10017

POA ACCEPTANCE LETTER

OC000000037288199

Date Mailed: 08/12/2009

NOTICE OF ACCEPTANCE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 08/05/2009.

The Power of Attorney in this application is accepted. Correspondence in this application will be mailed to the above address as provided by 37 CFR 1.33.

/ttkim/

Office of Data Management, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
12/466,213	05/14/2009	Jeffrey B. Etter	9516-847-999

CONFIRMATION NO. 5370

84802
JONES DAY
222 E. 41ST. STREET
NEW YORK, NY 10017

PUBLICATION NOTICE

OC000000038861113

Title:ORAL FORMULATIONS OF CYTIDINE ANALOGS AND METHODS OF USE THEREOF

Publication No.US-2009-0286752-A1
Publication Date:11/19/2009

NOTICE OF PUBLICATION OF APPLICATION

The above-identified application will be electronically published as a patent application publication pursuant to 37 CFR 1.211, et seq. The patent application publication number and publication date are set forth above.

The publication may be accessed through the USPTO's publically available Searchable Databases via the Internet at www.uspto.gov. The direct link to access the publication is currently http://www.uspto.gov/patft/.

The publication process established by the Office does not provide for mailing a copy of the publication to applicant. A copy of the publication may be obtained from the Office upon payment of the appropriate fee set forth in 37 CFR 1.19(a)(1). Orders for copies of patent application publications are handled by the USPTO's Office of Public Records. The Office of Public Records can be reached by telephone at (703) 308-9726 or (800) 972-6382, by facsimile at (703) 305-8759, by mail addressed to the United States Patent and Trademark Office, Office of Public Records, Alexandria, VA 22313-1450 or via the Internet.

In addition, information on the status of the application, including the mailing date of Office actions and the dates of receipt of correspondence filed in the Office, may also be accessed via the Internet through the Patent Electronic Business Center at www.uspto.gov using the public side of the Patent Application Information and Retrieval (PAIR) system. The direct link to access this status information is currently http://pair.uspto.gov/. Prior to publication, such status information is confidential and may only be obtained by applicant using the private side of PAIR.

Further assistance in electronically accessing the publication, or about PAIR, is available by calling the Patent Electronic Business Center at 1-866-217-9197.

Office of Data Managment, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

LIST OF REFERENCES CITED BY APPLICANT (Use several sheets if necessary)		
	Application Number	12/466,213
	Filing Date	May 14, 2009
	First Named Inventor	Etter et al.
	Art Unit	1623
	Examiner Name	To be determined
	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
	A01	US 20010026807	10/04/2001	Watts	
	A02	US 20020051820	05/02/2002	Shell et al.	
	A03	US 6,432,924	08/13/2002	Nyce	
	A04	US 20030039688	02/27/2003	Shell et al.	
	A05	US 20030049311	03/13/2003	McAllister et al.	
	A06	US 20030104053	06/05/2003	Gusler et al.	
	A07	US 20030104062	06/05/2003	Berner et al.	
	A08	US 20030220254	11/27/2003	Khan et al.	
	A09	US 20040162263	08/19/2004	Sands et al.	
	A10	US 6,887,855	05/03/2005	Ionescu et al.	
	A11	US 6,890,547	05/10/2005	Takada et al.	
	A12	US 6,943,249	09/13/2005	Ionescu et al.	
	A13	US 20050272675	12/08/2005	Ionescu et al.	
	A14	US 7,078,518	07/18/2006	Ionescu et al.	
	A15	US 20060247189	11/02/2006	Ionescu et al.	
	A16	US 20070270374	11/22/2007	Gallop	
	A17	US 20080057086	03/06/2008	Etter	

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
	B01	WO 2008/028193	03/06/2008	Etter		
	B02	WO 2009/052287	04/23/2009	Hamilton *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
	C01	APARICIO et al., Current Opinion in Investigational Drugs, 2002, 3(4), 627–33.	
	C02	ARGEMI et al., Journal of Pharmaceutical and Biomedical Analysis, 2007, 44, 859–66.	

SDI-12640v1

EXAMINER SIGNATURE	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.

LIST OF REFERENCES CITED BY APPLICANT (Use several sheets if necessary)		
	Application Number	12/466,213
	Filing Date	May 14, 2009
	First Named Inventor	Etter et al.
	Art Unit	1623
	Examiner Name	To be determined
	Attorney Docket No.	9516-847-999

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
	C03	BEISLER et al., Journal of Medicinal Chemistry, 1977, 20(6), 806–12.	
	C04	BELLET et al., Cancer Chemotherapy Reports, Part I, 1974, 58(2), 217–22.	
	C05	BELLET et al., Medical and Pediatric Oncology, 1978, 4, 11–15.	
	C06	BHUYAN et al., Cancer Research, 1972, 32, 398–407.	
	C07	BHUYAN et al., Cancer Research, 1973, 33, 888–94.	
	C08	BROCK et al., New England Journal of Medicine, 2008, 358(11), 1118–28.	
	C09	CHAN et al., Journal of Pharmaceutical Sciences, 1979, 68(7), 807–12.	
	C10	CHRISTMAN et al., Oncogene, 2002, 21, 5483–95.	
	C11	CUNNINGHAM et al., Cancer Chemotherapy Reports, Part I, 1974, 58(5), 677–81.	
	C12	CURT et al., Cancer Research, 1985, 45, 3359–63.	
	C13	DAS et al., Molecular Cancer, 2006, 5(28), doi: 10.1186/1476-4598-5-28, *available at* http://www.molecular-cancer.com/content/5/1/28.	
	C14	DOVER et al., Blood, 1985, 66(3), 527–32.	
	C15	FENAUX et al., The Lancet Oncology, 2009, 10(3), 223–32.	
	C16	GARCIA-MANERO et al., Current Opinion in Oncology, 2008, 20, 705–10.	
	C17	GIFFORD et al., Clinical Cancer Research, 2004, 10, 4420–26.	
	C18	ISRAILI et al., Cancer Research, 1976, 36, 1453–61.	
	C19	JORDAN et al., New England Journal of Medicine, 2006, 355(12), 1253–61.	
	C20	JUBB, et al., Journal of Pathology, 2001, 195, 111–34.	
	C21	KORNBLITH et al., Journal of Clinical Oncology, 2002, 20(10), 2441–52.	
	C22	LI et al., Cancer Research, 1970, 30, 2770–75.	
	C23	LIN et al., Journal of Pharmaceutical Sciences, 1981, 70(11), 1228–32.	
	C24	LOMEN et al., Cancer Chemotherapy Reports, Part I, 1975, 59(6), 1123–26.	
	C25	MOERTEL et al., Cancer Chemotherapy Reports, Part I, 1972, 56(5), 649–52.	
	C26	MOJAVERIAN et al., Journal of Pharmacy and Pharmacology, 1984, 36, 728–33.	
	C27	NEIL et al., Cancer Chemotherapy Reports, Part I, 1975, 59(3), 459–65.	
	C28	NOTARI et al., Journal of Pharmaceutical Sciences, 1975, 64(7), 1148–57.	
	C29	QUAGLIANA et al., Cancer Treatment Reports, 1977, 61(1), 51–54.	
	C30	SANDERSON et al., Nature News, March 16, 2009, *available at* http://www.nature.com/news/2009/090316/full/458269a.html.	
	C31	SHNIDER et al., Journal of Clinical Pharmacology, 1976, 205–12.	
	C32	SILVERMAN et al., Journal of Clinical Oncology, 2002, 20(10), 2429–40.	

EXAMINER SIGNATURE	**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.

LIST OF REFERENCES CITED BY APPLICANT (Use several sheets if necessary)		
	Application Number	12/466,213
	Filing Date	May 14, 2009
	First Named Inventor	Etter et al.
	Art Unit	1623
	Examiner Name	To be determined
	Attorney Docket No.	9516-847-999

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
	C33	SILVERMAN et al., Journal of Clinical Oncology, 2006, 24(24), 3895–3903.	
	C34	SKIKNE et al., "A Phase I, Open-Label, Dose-Escalation Study to Evaluate the Safety, Pharmacokinetics, and Pharmacodynamics of Oral Azacitidine in Subjects with Myelodysplastic Syndromes (MDS) or Acute Myelogenous Leukemia (AML)," Journal of Clinical Oncology (May 20, 2008 Supplement), 2008 ASCO Annual Meeting Proceedings (Meeting Date: May 30–June 3, 2008), Part I, 2008, 26(15S), poster # 7091.	
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	C41	International Search Report, for PCT/US2009/002999, filed May 14, 2009.	

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(54) Title: COLON-TARGETED ORAL FORMULATIONS OF CYTIDINE ANALOGS

(57) Abstract: The present invention provides an oral formulation of a cytidine analog, including, 5-azacytidine, for delivery to the lower gastrointestinal tract, including, the large intestine; methods to treat diseases associated with abnormal cell proliferation by treatment with the oral formulations of the present invention; and methods to increase the bioavailability of a cytidine analog upon administration to a patient by providing an oral formulation of the present invention.

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COLON-TARGETED ORAL FORMULATIONS OF CYTIDINE ANALOGS

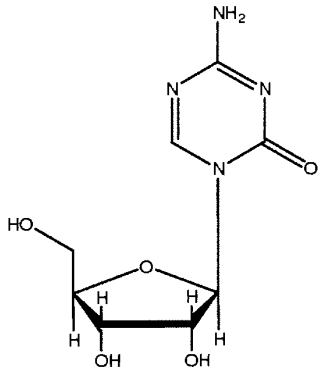
BACKGROUND OF THE INVENTION

[0001] Cellular proliferative disorders are responsible for numerous diseases resulting in major morbidity and mortality and have been intensively investigated for decades. Cancer now is the second leading cause of death in the United States, and over 500,000 people die annually from this proliferative disorder.

[0002] 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.

[0003] 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 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 molecular formula of $C_8H_{12}N_4O_5$, a relative molecular weight of 244.21 and a structure of:

[0004]



[0005] Azacitidine (also referred to herein as 5-azacytidine 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 oxygen.

[0006] Other members of the class of deoxycytidine and cytidine analogs include arabinosylcytosine (Cytarabine), 2'-deoxy-2',2'-difluorocytidine (Gemcitabine), 5-aza-2'-deoxycytidine (Decitabine), 2(1H) 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).

[0007] In general, oral delivery of members of this class of compounds has proven difficult due to combinations of chemical instability, enzymatic instability, and/or poor tissue permeability. For example, these compounds are known to be acid labile and thus unstable in the acidic gastric environment. In the case of 5-azacytidine, ara-C, decitabine and gemcitabine, an enzyme thought to be responsible for a significant portion of drug metabolism is cytidine deaminase. Strategies to improve the oral bioavailability of this drug class have included the use of prodrugs to modify chemical and enzymatic instability, and/or the use of enzymatic inhibitors.

[0008] For example, DeSimone et al describe the ability of 5-azacytidine to induce fetal hemoglobin production in baboons when administered via the intravenous (IV), subcutaneous (SC), or perioral (PO) route. In the case of PO administration the author states that co-administration of THU (tetrahydrouridine) was necessary to achieve fetal hemoglobin induction, however no specific data is provided on the doses or responses observed without THU. 5-azacytidine doses ranged from 0.25 mg/kg/d to 8 mg/kg/d with co-administration of 20 mg/kg/d THU. Administration of THU alone was shown to result in a significant decrease in peripheral cytidine deaminase activity.

[0009] Neil, et al describe the effect of THU on the pharmacokinetics and pharmacodynamics of inter peritoneal (I.P.) and peri oral (P.O.) 5-azacytidine when administered to leukemic mice. Pharmacokinetic parameters were determined using a bioassay that did not discriminate between 5-azacytidine and its degradation and metabolism products. Inclusion of

THU with IP administration had little effect on the clearance or degradation of 5-azacytidine. Inclusion of THU with PO administration significantly increased both C_{max} and $t_{1/2}$. In both acute and chronic IP dosing the inclusion of THU did not influence the pharmacodymamic effects of 5-azacytidine except at the highest chronic dose which was toxic. Conversely, co-administration of THU with PO 5-azacytidine resulted in increased efficacy at all doses except the highest chronic dose which was again toxic.

[0010] Dunbar, et al describe the administration of 5-azacytidine via IV and PO routes for increased production of total hemoglobin in a β^0-thalassemic patient. Doses of 2 mg/kg/d IV resulted in a measurable increase to hemoglobin levels. Administration of 2 mg/d tid (three times daily) PO with co-administration of THU did not result in increased hemoglobin levels.

[0011] Dover, et al describe administration of 5-azacytidine via the SC and PO routes for increased production of total hemoglobin, fetal hemoglobin and F cells in sickle cell patients. 5-azacytidine oral bioavailability was assessed by clinical response only. Dover reports that oral doses of 5-azacytidine (2 mg/kg/d) alone or THU (200 mg/d) alone did not result in increased F reticulocyte production. However oral doses of 200 mg/d of THU were observed to result in a significant suppression of peripheral cytidine deaminase activity for several days post administration. When 5-azacytidine was co-administered with THU good clinical response was observed as determined by total hemoglobin, fetal hemoglobin and F cell levels. In fact comparable clinical response was observed with doses of 2 mg/kg/d SC without THU versus 0.2 mg/kg/d PO with co-administration of 200 mg/d THU. Oral doses of 5-azacytidine and THU were prepared by encapsulation at the clinical site. No information was provided with respect to excipients.

[0012] Efforts to increase bioavailability of this class of compounds have also been described in, for example, U.S. Patent Application Publication No. 2004/0162263 (Sands, et al.) In this publication, delivery of 5-azacytidine in an enteric-coated formulation are disclosed such that the drugs are preferably absorbed in the upper regions of the small intestine, such as the jejunum. All U.S. patents and patent publications referenced herein are incorporated by reference herein in their entireties.

[0013] Despite these efforts, a need remains for more effective methods and compositions which increase oral bioavailability of this class of compounds.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] Figure 1 represents a graph showing Absolute Mucosal to Serosal Permeability of 5-azacytidine in Human Intestinal Tissue with and without Enzymatic Inhibition.

[0015] Figure 2 represents a graph showing Relative Mucosal to Serosal Permeability of 5-azacytidine in Human Intestinal Tissue with and without Enzymatic Inhibition with Respect to Atenolol.

[0016] Figure 3 represents a graph showing Absolute Mucosal to Serosal Permeability of 5-azacytidine in Human Colonic Tissue with Various Concentrations of TPGS or Labrafil without Enzymatic Inhibition.

[0017] Figure 4 represents a graph showing Relative Mucosal to Serosal Permeability of 5-azacytidine in Human Colonic Tissue with Various Concentrations of TPGS or Labrafil without Enzymatic Inhibition.

[0018] Figure 5 shows concentration vs time profiles of individual subjects administered an oral formulation of the present invention.

[0019] Figure 6 shows concentration vs time profiles for the 60 mg dose and the mean of the three 80 mg doses for individual subjects administered an oral formulation of the present invention.

SUMMARY OF THE INVENTION

[0020] In a first embodiment, the present invention comprises a controlled release pharmaceutical composition for oral administration for enhanced systemic delivery of a cytidine analog comprising a therapeutically effective amount of a cytidine analog and a drug release controlling component which is capable of providing release of the cytidine analog primarily in the large intestine. After ingestion by a patient, the cytidine analog is released primarily in the large intestine.

[0021] In another embodiment, the present invention includes a method for treating a patient having a disease associated with abnormal cell proliferation. The method includes orally administering to the patient a controlled release pharmaceutical composition, comprising a therapeutically effective amount of a cytidine analog and a drug release controlling component which is capable of providing release of the cytidine analog primarily in the large intestine. After ingestion by a patient the cytidine analog is released primarily in the large intestine.

[0022] In another embodiment, the present invention includes a method of increasing the bioavailability of a cytidine analog upon administration to a patient, comprising the following steps. First, provided is a controlled release pharmaceutical composition, comprising a therapeutically effective amount of a cytidine analog and a drug release controlling component capable of providing release of the cytidine analog primarily in the large intestine. Second, the patient ingests the composition, whereupon the composition contacts the biological fluids of the patient's body and increases the bioavailability of the cytidine analog.

[0023] In one embodiment, a condition to treat using the present invention is a myelodysplastic syndrome. In one embodiment, the cytidine analog is 5-azacytidine. In one embodiment, the drug release controlling component is an enteric coating.

DETAILED DESCRIPTION OF THE INVENTION

[0024] The present invention is based on the surprising discovery that 5-azacytidine and related compounds are best absorbed in the lower gastrointestinal tract, i.e., the large intestine (colon). Conventionally, it is expected that the upper gastrointestinal tract is the more desirable location for absorption, due to greater surface area, relatively greater liquidity, and the fact that typically the greater part of absorption of nutrients takes place therein. However, the inventors have found that in the case for cytidine analogs, absorption is greatest and most consistent between patients in colonic tissue. Accordingly, the present invention demonstrates the preparation of a solid oral dosage form of a cytidine analog, such as 5-azacytidine, using common pharmaceutical excipients designed for delivering pharmaceutical compositions to the large intestine and colon. The term "absorb", "absorption", "absorbed" and the like are used to indicate transfer of a cytidine analog across a relevant tissue, such as, for example, intestinal tissue. In some embodiments, absorbed cytidine analogs are taken up by the blood stream making the cytidine analog available at least partially systemically. In some embodiments, absorption occurs without substantive degradation (i.e., undesirable chemical modification of) of the cytidine analog.

[0025] Furthermore, the inventors have demonstrated that inclusion of THU (taught by others as a requirement to facilitate bioavailability of this drug class) is not necessary to achieve useful oral bioavailability of cytidine analogs via delivery in the large intestine and colon. Accordingly, formulations of the present invention obviate the need to utilize enzymatic

inhibitors such as THU in formulations to increase bioavailability of cytidine analogs. Avoidance of enzymatic inhibitors is a desirable attribute for a therapeutic dosage form since such inclusion increases the formulation cost and complexity, and may result in instability, or undesirable, pharmacological, toxicological or other effects. Accordingly, oral delivery of 5-azacytidine without inclusion of an enzymatic inhibitor is possible when the target tissue to which the drug is delivered is the colon. In the case of PO delivery of 5-azacytidine to humans, data suggests that delivery to the upper GI tract may well benefit from enzymatic inhibition, however delivery to the colon does not require the inclusion of such an inhibitor. Targeting to the colon may be achieved with commercially available and pharmaceutically acceptable coatings such as, for example, enteric coatings.

[0026] Furthermore, the inventors have demonstrated the preparation of solid oral dosage forms containing excipients and coatings which possess acceptable production and stability characteristics for use as a pharmaceutical dosage form.

[0027] In one embodiment, the present invention includes a controlled release pharmaceutical composition for oral administration comprising a) a therapeutically effective amount of a cytidine analog and b) a drug release controlling component for providing the release of the cytidine analog primarily in the large intestine. The controlled release pharmaceutical compositions of the present invention will in one embodiment lack THU.

[0028] In one embodiment, the cytidine analog useful in the present invention 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 analogs to use with the present invention include 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(1H) pyrimidine riboside (also called zebularine), 2'-cyclocytidine, arabinofuanosyl-5-azacytidine, dihydro-5-azacytidine, N^4-octadecyl-cytarabine, and elaidic acid cytarabine. In one embodiment, is 5-azacytidine and 5-aza-2'-deoxycytidine The definition of cytidine analog used herein also includes mixtures of cytidine analogs.

[0029] Cytidine analogs useful in the present invention may be manufactured by any methods known in the art. In one embodiment, methods to manufacture 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.

[0030] In one embodiment, the amounts of a cytidine analog to use in methods of the present invention and in the oral formulations of the present invention include a therapeutically effective amount. Therapeutic indications are discussed more fully herein below. Precise amounts for therapeutically effective amounts of the cytidine analog in the pharmaceutical compositions of the present invention 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 150 mg/m^2(based on patient body surface area) or about 4 mg/kg (based on patient body weight) as single or divided (2-3) daily doses.

[0031] The controlled release pharmaceutical compositions of the present invention include a drug release controlling component. The drug release controlling component is adjusted such that the release of the cytidine analog occurs primarily in the large intestine. In one embodiment, at least about 95% of the cytidine analog is released in the large intestine, or at least about 90% of the cytidine analog is released in the large intestine. In other embodiments, at least about 80% of the cytidine analog is released in the large intestine, at least about 70% of the cytidine analog is released in the large intestine, at least about 60% of the cytidine analog is released in the large intestine, or at least about 50% of the cytidine analog is released in the large intestine. In other embodiments, the amount released in the intestines is at least about 40%, at least about 30%, or at least about 20% of the cytidine analog. The term "release" refers to the process whereby the cytidine analog is made available for uptake by or transport across the epithelial cells that line the large intestine and is made available to the body.

[0032] The pharmaceutical compositions of the present invention are intended for oral delivery. Oral delivery includes formats such as tablets, capsules, caplets, solutions, suspensions and/or 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 as the "drug core" which contains the cytidine analog. Such dosage forms are prepared using conventional methods known to those in the field of pharmaceutical formulation and are described in the pertinent

texts, e.g., in REMINGTON: THE SCIENCE AND PRACTICE OF PHARMACY, 20th Edition, Lippincott Williams & Wilkins, 2000).

[0033] Tablets and capsules represent the most convenient oral dosage forms, in which case solid pharmaceutical carriers are employed. Tablets are used in one embodiment. Tablets may be manufactured using standard tablet processing procedures and equipment. One method for forming tablets is by direct compression of a powdered, crystalline or granular composition containing the cytidine analog, alone or in combination with one or more carriers, additives, or the like. As an alternative to direct compression, tablets can be prepared using wet-granulation or dry-granulation processes. Tablets may also be molded rather than compressed, starting with a moist or otherwise tractable material; particularly, compression and granulation techniques are used in one embodiment.

[0034] In another embodiment, capsules may be used. Soft gelatin capsules may be prepared in which capsules contain a mixture of the active ingredient and vegetable oil or non-aqueous, water miscible materials such as, for example, polyethylene glycol and the like. Hard gelatin capsules may contain granules of the active ingredient in combination with a solid, pulverulent carrier, such as, for example, lactose, saccharose, sorbitol, mannitol, potato starch, corn starch, amylopectin, cellulose derivatives, or gelatin. A hard gelatin capsule shell can be prepared from a capsule composition comprising gelatin and a small amount of plasticizer such as glycerol. As an alternative to gelatin, the capsule shell may be made of a carbohydrate material. The capsule composition may additionally include colorings, flavorings and opacifiers as required.

[0035] The cytidine analog in one embodiment is prepared as a controlled release tablet or capsule which includes a drug core comprising the pharmaceutical composition and optional excipients (described elsewhere herein). Optionally, a "seal coat", described elsewhere herein, is applied to the drug core before addition of the drug release component. The drug release component is formulated to provide for release of the cytidine analog primarily in the large intestine (colon). In one embodiment, minimal release of the cytidine analog occurs in the upper reaches of the gastrointestinal tract, e.g., the stomach and small intestine.

[0036] The small intestine extends from the pylorus to the colic valve where it ends in the large intestine. The small intestine is about 6 meters long and is divisible into three portions: the duodenum, the jejunum, and the ileum. The small intestine is especially adapted for transport

and absorption of nutrients and other molecules from ingested material, passing through the lining of the small intestine into the blood. The surface cells of the small intestine are highly specialized for digestion and absorption of nutrients. Almost all the body's nutrient absorption occurs in the small intestine, along its three sub-divisions: the duodenum, jejunum, and ileum. Sites for absorption of specific nutrients (eg: iron, vitamin.B12) are located in these divisions, but most absorption occurs in the jejunum (middle section). Specialized cells contain digestive enzymes, carrier proteins and other secretions. Blood vessels transport nutrients away from the intestine to the liver in the first instance.

[0037] Indigestible food passes into the large intestine. By the time ingested material leaves the small intestine, virtually all nutrient absorption will have occurred. The large intestine extends from the end of the ileum (distal ileum) to the anus. The large intestine is divided into the cecum, colon, rectum, and anal canal. The colon is divided into four parts: the ascending, transverse, descending, and sigmoid. The substantial release of the cytidine compound of the present invention may occur in any portion of the large intestine. In one embodiment, release primarily occurs at the upper regions of the large intestine, such as, for example, at the distal ileum, cecum, and/or the ascending colon.

[0038] It is known that there are major variations in acidity in the gastrointestinal tract. The stomach is a region of high acidity (about pH 1 to 3). Specific glands and organs emptying into the small intestine raise the pH of the material leaving the stomach to approximately pH 6.0 to 6.5. The large intestine and the colon are about pH 6.4 to 7.0. The transit time through the small intestine is approximately three hours. In contrast, the transit time through the large intestine is approximately 35 hours.

[0039] Methods by which to formulate compositions to target specific regions of the gastrointestinal tract are known in the art, described in numerous publications, and all references specifically cited within the present document are incorporated by reference herein. For example, release of drug in the gastrointestinal tract may be accomplished by choosing a drug release controlling component to work together with some physical, chemical or biochemical process in the gastrointestinal tract. A drug release controlling component may take advantage of processes and/or conditions within the gastrointestinal tract and in specific regions of the gastrointestinal tract such as, for example, osmotic pressure, hydrodynamic pressure, vapor pressure, mechanical action, hydration status, pH, bacterial flora, and enzymes. Specific U.S.

Patents incorporated by reference herein include, among others, U.S. Pat. No. 3,952,741, U.S. Pat. No. 5,464,633, U.S. Pat. No. 5,474,784, U.S. Pat. No. 5,112,621.

[0040] Optionally, pharmaceutical compositions of the present invention including drug cores may further comprise a seal coating material that seals the drug to prevent decomposition due to exposure to moisture, such as hydroxylpropylmethylcellulose. Accordingly, the drug core of the pharmaceutical composition (containing the cytidine analog) may first be sealed with the seal coating material and then coated with the drug release controlling component to prevent decomposition of the cytidine analog by exposure to moisture. Seal coating materials include, in one embodiment, acetyltributyl citrate, acetyltriethyl citrate, calcium carbonate, carauba wax, cellulose acetate, cellulose acetate phthalate, cetyl alcohol, chitosan, ethylcellulose, fructose, gelatin, glycerin, glyceryl behenate, glyceryl palmitostearate, hydroxyethyl cellulose, hydroxyethylmethyl cellulose, hydroxypropyl cellulose, hypromellose, hypromellose phthalate, isomalt, latex particles, maltitol, maltodextrin, methylcellulose, microcrystalline wax, paraffin, poloxamer, polydextrose, polyethylene glycol, polyvinyl acetate phthalate, polyvinyl alcohol, povidone, shellac, shellac with stearic acid, sodium carboxymethyl cellulose, sucrose, titanium oxide, tributyl citrate, triethyl citrate, vanillin, white wax, xylitol, yellow wax, and zein. Compositions of the present invention may also include film forming agents, which include, for example, ammonium alginate, calcium carbonate, chitosan, chlorpheniramine maleate, copovidone, dibutyl phthalate, dibutyl sebacate, diethyl phthalate, dimethyl phthalate, ethyl lactate, ethylcellulose, gelatin, hydroxyyethyl cellulose, hydroxypropyl cellulose, hypromellose, hypromellose acetate succinate, maltodextrin, polydextrose, polyethylene glycol, polyethylene oxide, polymethylacrylates, poly(methylvinyl ether/maleic anhydride), polyvinylacetate phthalate, triethyl citrate, and vanillin. The amount of seal coating will vary in accordance with factors known by those of skill in the art. The amount of seal coat is, in one embodiment, 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 11%, 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; or more, if determined to be appropriate. Seal coats may also be applied at amounts between about 1% and about 10% w/w

of the drug core, between about 2% and 9% w/w of the drug core, between about 3% and 8% w/w of the drug core, between about 4% and 7% w/w of the drug core, and between about 5% and about 6% w/w of the drug core.

[0041] In one embodiment, drug release controlling components include, for example, coatings, matrices, or physical changes. Coatings are used in one embodiment. Coatings include, for example, enteric coatings, time delay coatings, bacterially degradable coatings, and mixtures thereof. The pharmaceutical composition may comprise multiple coatings of either the same or different types of coatings. In choosing an appropriate coating or mixture thereof, the formulations practitioner may consider a number of variables influencing the location in which a drug will become available in the gastrointestinal tract, e.g., the pH at which coatings dissolve; the time of dissolution (which is influenced by thickness of the coatings and/or additional components in the coatings); time of transit through the gastrointestinal tract, and whether the coatings can be degraded by the patent's digestive enzymes or require enzymes present only in bacteria residing in the lower intestine. As an example of a combination drug release controlling component is, for example, an inner core with two polymeric layers. The outer layer, an enteric coating, may be chosen to dissolve at a pH level above 5. The inner layer, may be made up of hydroxypropylmethylcellulose to act as a time delay component to delay drug release for a predetermined period. The thickness of the inner layer can be adjusted to determine the lag time.

[0042] Methods by which skilled practitioners can assess where a drug is released in the gastrointestinal tract of either animal models or human volunteers are known in the art, and include scintigraphic studies, testing in biorelevant medium which simulates the fluid in relevant portions of the gastrointestinal tract, among others.

[0043] In one embodiment, a drug release controlling component may include an enteric coating. The term "enteric coating" refers to a coating that allows a cytidine analog formulation to pass through the stomach substantially intact and subsequently disintegrate substantially in the intestines. In one embodiment, the disintegration occurs in the large intestine.

[0044] The coating of pH-sensitive (enteric) polymers to tablets, capsules and other oral formulations of the present invention provided delayed release and protect the active drug from gastric fluid. In general, enteric coatings should be able to withstand the lower pH values of the stomach and small intestine and be able to disintegrate at the neutral or slightly alkaline pH of the large intestine. Enteric coatings are a well known class of compounds. Coating

pharmaceutically active compositions with enteric coatings is well known in the art to enable pharmaceutical compositions to bypass the stomach and its low acidity. Enteric coatings generally refer to a class of compounds that dissolve at or above a particular pH and include a number of pH-sensitive polymers. The pH dependent coating polymer may be selected from those enteric coatings known to those skilled in the art. Such polymers may be one or more of the group comprising hydroxypropylmethylcellulose phthalate, polyvinyl acetate phthalate (PVAP), hydroxypropylmethylcellulose acetate succinate (HPMCAS), alginate, carbomer, carboxymethyl cellulose, methacrylic acid copolymer (such as, for example, a cationic copolymer of dimethyl aminoethyl methacrylate and neutral methacrylic esters), polyvinyl acetate phthalate, cellulose acetate trimellitate, shellac, cellulose acetate phthalate (CAP), starch glycolate, polacrylin, methyl cellulose acetate phthalate, hydroxylmethylcellulose phthalate, hydroxymethylmethylcellulose acetate succinate, hydroxypropylcellulose acetate phthalate, cellulose acetate terephthalate, cellulose acetate isophthalate, and includes the various grades of each polymer such as HPMCAS-LF, HPMCAS-MF and HPMCAS-HG, or mixtures thereof. Other enteric coatings suitable for the present invention include acetyltributyl citrate, carbomers, guar gum, hypromellose acetate succinate, hypromellose phthalate, polymethacrylates, tributyl citrate, triethyl citrate, white wax, and zein.

[0045] In one embodiment, the pH dependent coating is selected from the group consisting of methacrylic acid copolymers of varying threshold pH (such as, but not limited to EUDRAGIT S 100 (a cationic copolymer of dimethyl aminoethyl methacrylate and neutral methacrylic acid esters manufactured by Rohm Pharma GmbH of Darmstadt, Germany)).

[0046] Multiple coatings of enteric polymers may be utilized. In one embodiment, the first coating (closest to the core) is an enteric coating that will survive until the dosage form arrives at the large intestine/colon. To target the large intestine, in one embodiment an enteric coating comprises a series of methacrylic acid anionic copolymers known as EUDRAGIT S. The EUDRAGIT S films are colorless, transparent and brittle. In one embodiment, the enteric coating comprises EUDRAGIT S100. The EUDRAGIT S coatings are insoluble in pure water, in buffer solutions below a pH of 6.0 and also in natural and artificial gastric juices. They are slowly soluble in the region of the digestive tract where the juices are neutral to weakly alkaline (i.e., the large intestine and the colon) and in buffer solutions above a pH of 7.0. Mixtures of these various enteric polymers recited above, can be used in the present invention. Further, the

use of plasticizers is included in one embodiment with the enteric polymer coatings useful herein.

[0047] As known in the art and discussed in sources such as Patel et al. "Colon Specific Delivery" *Drug Delivery Technology* (2006) Vol. 6 62-71, and Khan et al., J. *Controlled Release* 1999; 58:215-222, the disintegration rates of enteric coated tablets are dependent on the polymer combination used to coat the tablets, the pH of the disintegration media, and the coating level of the tablets (i.e., thickness of the coating). The presence of plasticizer and the nature of the salts in the dissolution medium also influence the dissolution rate. A number of specific formulations effective for release in the colon in human volunteers, using *in vivo* scintigraphic studies, is disclosed in Patel et al., and are incorporated by reference herein.

[0048] The enteric coating may also be modified through the inclusion of an edible acid to retard or slow the dissolution of the coating in the intestines. Any edible acid may be used. Representative edible acids include acetic acid, benzoic acid, fumaric acid, sorbic acid, propionic acid, hydrochloric acid, citric acid, malic acid, tartaric acid, isocitric acid, oxalic acid, lactic acid, the phosphoric acids and mixtures thereof. One embodiment includes fumaric acid and malic acids. The weight percent of the edible acid in the enteric coating solution (polymer, plasticizer, anti-tack agents, water and the like) can range from about 5 to about 40%, with 10 to 30% present in one embodiment and 10 to 25% in another embodiment. Those skilled in the art will readily be able to determine the exact amount of edible acid to include in the coating solution, depending upon the pKa of the particular edible acid and the desired delay in dissolution of the enteric coating. After application of the enteric coating solution, as further described below, the percent of edible acid in the coating will range from about 10 to about 80 weight % of the coating; 20 to 60% in one embodiment; and 25-50% in another.

[0049] Enteric coatings can be obtained from a number of manufacturers, such as, for example, Rohm Pharma GmbH of Darmstadt, Germany (EUDRAGIT). Particular blends of pH sensitive polymers and types can be selected by one of skill in the art. As an example, the manufacturer of EUDRAGIT polymers teaches that the EUDRAGIT grades for sustained release formulations are based on copolymers of acrylate and methacrylates with quaternary ammonium groups as functional groups as well as ethylacrylate methylmethacrylate copolymers with a neutral ester group. EUDRAGIT polymers are available insoluble and/or permeable. For example, the EUDRAGIT RL-types are highly permeable, the EUDRAGIT RS-types are poorly

permeable, the EUDRAGIT NE-types are swellable and permeable. The release profiles and locations of release can be determined by varying mixing ratios of the polymers and/or film thickness of the coatings and such profiles can be adjusted by those of skill in the art.

[0050] In some embodiments, coatings include those that selectively dissolve at a pH at or above the pH generally prevailing in the large intestine, for example, above about pH 6, above about pH 6.2, above about pH 6.4, above about pH 6.6, above about pH 6.8, or above about pH 7. In one embodiment, the enteric coating will selectively dissolve in the pH range of about 6.0 to about 7.5, in the pH range of about 6.2 to about 7.5, in the pH range of about 6.4 to about 7.2, in the pH range of about 6.5 to about 7, in the pH range of about 6.5 to 6.8. As an example of coatings and their "threshold" pH (the pH at which the coating will dissolve) which the skilled practitioner may consider include, but are not limited to, cellulose phthalates (e.g, hydropropylmethylcellulose phthalates (HPMCPs)) that selectively dissolve at pH above 5.6, the EUDRAGIT family of polymers which are anionic polymer based on methacrylic acid and methacrylates with carboxyl functional groups (e.g., EUDRAGIT L30D with threshold pH of 5.6, EUDRAGIT L with threshold pH of 6.0, and EUDRAGIT S with threshold pH of 6.8), AQUATERIC with threshold pH of 5.8, polyvinylacetate phthalate (PVAP) that releases drug at pH values above about 5.0, shellac that is obtained from a gummy exudation produced by female insects, *Laccifer lacca kerr*, and releases drug at about pH 7.0, and cellulose acetate phthalate (CAP) with threshold pH of 6.0. In a one embodiment, the drug is enteric-coated with EUDRAGIT S100 with threshold pH of 7.0, which will degrade measurably at slightly lower pH such as pH 6.8.

[0051] In one embodiment, prior to application to the tablets, capsules, or drug core of the present invention, the drug release controlling component, such as, for example, the enteric coatings useful in the present invention, will be dissolved in a non-aqueous solution in order to create the solid oral formulation of the present invention. Examples of such non aqueous solutions include any known in the art suitable for pharmaceutical formulation procedures, including, for example, acetone-isopropanol solvent mixtures, methylene chloride-ethanol solvent mixtures, acetone-ethanol solvent mixtures, benzene-methanol solvent mixtures, acetate-ethanol solvent mixtures, among others. Proportions of each solvent to use and conditions will be readily determined by those of skill in the art. The solid dispersion of the composition of the present invention, in one embodiment, can be formed by spray drying techniques, although it

will be understood that suitable solid dispersions may be formed by a skilled addressee utilizing other conventional techniques, such as co-grinding, melt extrusion, freeze drying, rotary evaporation or any solvent removal process. In one embodiment, spray drying is utilized. The enteric coating may be applied over the entire surface area or portions thereof. In one embodiment, the entire surface area is coated.

[0052] In one embodiment, the enteric coat comprises EUDRAGIT S100 and the amount of enteric coat to use, relative to the drug core, or additional to the drug core, an 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; 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 91%, 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. Ranges include between about 2% and about 20% w/w additional; between about 3% and about 15% w/w additional; between about 4% and about 10% w/w additional; between about 5% and about 9% w/w additional; between about 6% and about 8% w/w additional.

[0053] As referenced elsewhere herein, enteric coats (and any drug release controlling component of the present invention, including time delay and bacterially degradable coats, and mixtures thereof) may also optionally further comprise a plasticizer. If a plasticizer is present, the drug release controlling component may be present in an amount of about 1% w/w of the coat, about 2% w/w of the coat; about 3% w/w of the coat; about 4%, w/w of the coat, about 5% w/w of the coat; about 6%, w/w, of the coat, about 7% w/w of the coat, about 8%, w/w, of the coat; about 9% w/w of the coat, about 10% w/w of the coat; about 12%, w/w of the coat; about 14%, w/w, of the coat, about 16%, w/w/, of the coat; about 18% w/w of the coat; about 20%, w/w of the coat, about 22%, w/w, of the coat, about 24%, w/w, of the coat; about 26% w/w of the coat; about 28%, w/w of the coat, about 30%, w/w, of the coat, about 32%, w/w, of the coat; about 34% w/w of the coat; about 36%, w/w of the coat, about 38%, w/w, of the coat, about 40%, w/w, of the coat; about 42% w/w of the coat; about 44%, w/w of the coat; about 46%, w/w, of the coat, about 48%, w/w/, of the coat; about 50% w/w of the coat; about 52%, w/w of the coat, about 54%, w/w, of the coat, about 56%, w/w, of the coat; about 58% w/w of the coat; about 60%, w/w of the coat, about 62%, w/w, of the coat, about 64%, w/w, of the coat; about 66% w/w of the coat; about 68%, w/w of the coat, about 70%, w/w, of the coat, about 72%, w/w, of the coat; about 74% w/w of the coat; about 76%, w/w of the coat; about 78%, w/w, of the coat, about 80%, w/w/, of the coat; about 82% w/w of the coat; about 84%, w/w of the coat, about 86%, w/w, of the coat, about 88%, w/w, of the coat; about 90% w/w of the coat; about 92%, w/w of the coat, about 91%, w/w, of the coat, about 96%, w/w, of the coat; about 98%, w/w, of the coat, or more, if determined to be appropriate. Ranges include between about 30% and about 95% w/w of the coat; between about 40% and about 95% w/w of the coat; between about 50% and about 95% w/w of the coat; between about 60% and about 95% w/w of the coat; between about 70% and about 95% w/w coat.

[0054] In another embodiment, the amount of the enteric-coating material, when the coating material is EUDRAGIT S100, in one embodiment is about 1-10% w/w in the total composition, about 3-8% w/w in the total composition, or 4-7% w/w in the total composition. Appropriate amounts of coating to use will vary depending on the type of coating used, tablet size, surface preparation, target dissolution time at a given pH, etc. All things being equal, to determine the amount and/or thickness of the enteric coating, as the pH threshold of the enteric coating material increases, the relative amount and thickness of the coating can be decreased to

achieve dissolution of the tablet in a specified time frame at a particular pH. Routine empirical studies to determine the optimum conditions for targeting the large intestine may be carried out by the skilled person.

[0055] According to the invention, the controlled release pharmaceutical composition comprising a cytidine analog and an enteric coating, in one embodiment, will not substantially disintegrate in an acidic, aqueous medium at about pH 1-3 for at least one hour, at least two hours, or at least three hours. The composition is considered to be substantially disintegrated if at least 50% of the composition disintegrates, e.g., undergoes rupture and release. In some embodiments, the controlled release pharmaceutical composition comprising a cytidine analog and an enteric coating disintegrates substantially in an aqueous medium at about pH 7 or above within about ten hours, within about eight hours, within about six hours, within about four hours, within about two hours, within about one hour, within about 45 minutes, within about 30 minutes, and within about 15 minutes. In some embodiments, the controlled release pharmaceutical composition comprising a cytidine analog and an enteric coating disintegrates substantially in an aqueous medium at about pH 6.8 or above within about ten hours, within about eight hours, within about six hours, within about four hours, within about two hours, within about one hour, within about 45 minutes, within about 30 minutes, and within about 15 minutes. In some embodiments, times are within about two hours or less. In some embodiments, the controlled release pharmaceutical composition comprising a cytidine analog and an enteric coating does not disintegrate substantially in an aqueous medium at about pH 5 to about pH 6.5 for at least one hour, for at least about 1.5 hours, for at least about two hours, for at least about 2.5 hours, for at least about 3 hours, for at least about 3.5 hours, or for at least about four hours.

[0056] According to the invention, the controlled release pharmaceutical composition comprising a cytidine analog and an enteric coating, in one embodiment, will not substantially disintegrate after ingestion by a patient, on average, for at least about one hour, at least two hours, at least about three hours, at least about four hours, at least about five hours, at least about six hours, at least about seven hours, at least about eight hours, at least about nine hours, at least about ten hours, at least about twelve hours, at least about fourteen hours, at least about sixteen hours, at least about eighteen hours, at least about twenty hours, at least about twenty four hours, at least about twenty eight hours, or at least about thirty two hours. According to the invention,

the controlled release pharmaceutical composition comprising a cytidine analog and an enteric coating, in some embodiments, will substantially disintegrate after ingestion by a patient within about three hours, within about four hours, within about five hours, within about six hours, within about seven hours, within about eight hours, within about nine hours, within about ten hours, within about twelve hours, within about fourteen hours, within about sixteen hours, within about eighteen hours, within about twenty hours, within about twenty two hours, within about twenty four hours, within about twenty six hours, within about twenty eight hours, or within about thirty hours. The composition is considered to be substantially disintegrated if at least 50% of the composition disintegrates, e.g., undergoes rupture and release.

[0057] As examples of more specific types of controlled release pharmaceutical compositions, the following is described. For example, a pharmaceutical composition comprising a solid oral dosage form may be coated with a coating of a 60 to 150 micrometer thick layer of an anionic polymer which is insoluble in gastric juice and in intestinal fluid below pH 7 but soluble in colonic intestinal juice, so that the dosage form remains intact until the colon. For example, a pharmaceutical composition may be coated with a material which dissolves in the intestine and contained within a capsule which is also coated with a material which dissolves in the intestine. The composition is for selectively administering the drug to the intestine. The granules are in one embodiment contained within an enterically coated capsule which releases the granules in the small intestine and that the granules are coated with a coating which remains substantially intact until they reach at least the ileum and in one embodiment, thereafter provide a sustained or immediate release of the drug through the colon. Also there may be a non-disintegratable solid enteric pharmaceutical composition comprising a cytidine analog having relatively rapid dissolution at pH 6.5 from an excipient matrix, and dosage forms containing pellets of the composition. The rapid dissolution is increased by the presence of a rheological modifying super-disintegrant in an amount of at least 5% by weight, but insufficient to cause disintegration of the composition. It is stated that the composition may comprise a plurality of such pellets, which may be coated in an enteric coating such as cellulose acetate phthalate or, partly methyl esterified methacrylic acid polymers having a ratio of free acid groups to ester groups of about 1:2, contained in a capsule that is enterically coated with a suitable coating material. The coating material on the pellets is in one embodiment, one that is insoluble in gastric

juices and intestinal fluid below pH 7, but is soluble in the lower intestine. The enteric coating material of the capsule is chosen to protect the capsule during passage through the stomach.

[0058] In another embodiment, the pharmaceutical compositions of the present invention may include time delay coatings to delay release of the cytidine analog until reaching the large intestine. In accordance with this embodiment, solid dosage forms, whether tablets, capsules, caplets, or particulates, may, if desired, be coated so as to provide for delayed release. Sustained release dosage forms provide for drug release over an extended time period, and may or may not be delayed release. Generally, as will be appreciated by those of ordinary skill in the art, sustained release dosage forms are formulated by either dispersing a drug within a matrix of, a gradually bioerodible (hydrolyzable) material such as an insoluble plastic, a hydrophilic polymer, or a fatty compound, or by coating a solid, drug-containing dosage form with such a material. Insoluble plastic matrices may be comprised of, for example, polyvinyl chloride or polyethylene, vinyl polymers and copolymers such as polyvinyl pyrrolidone, polyvinyl acetate, polyvinylacetate phthalate, vinylacetate crotonic acid copolymer, and ethylene-vinyl acetate copolymers, zein, and shellac, ammoniated shellac, shellac-acetyl alcohol, and shellac n-butyl stearate. Fatty compounds for use as a sustained release matrix material include, but are not limited to, waxes generally (e.g., carnauba wax) and glyceryl tristearate.

[0059] In particular, time release coatings useful in the present invention may include the following: acetyltributyl citrate, acetyltriethyl citrate, aliphatic polyesters, bentonite, carbomers, carrageenan, cellulose acetate, cellulose acetate phthalate, ceratonia, cetyl alcohol, cetyl esters wax, chitosan, dibutyl sebacate, ethylcellulose, glycerin monostearate, glyceryl behenate, glyceryl monooleate, glyceryl monostearate, glyceryl palmitostearate, guar gum, hydroxypropyl cellulose, hypromellose acetate succinate, isopropyl palmitate, magnesium aluminum silicate, magnesium oxide, methylcellulose, microcrystalline wax, paraffin, peanut oil, potassium polacrilin, polycarbophil, polyethylene oxide, polymethacrylates, povidone, stearic acid, stearyl alcohol, talc, tributyl citrate, triethyl citrate, white wax, xanthan gum, yellow wax, and zein. Another useful time release coating is EUDRAGIT RS PO, copolymers of acrylic andmethacrylic acid esters with a low content in quaternary ammonium groups with an average molecular weight of about 150,000 D. This polymer has low permeability and water solubility with swelling that is pH-independent.

[0060] The type, amount and/or thickness of the matrix or coating can be readily adjusted by one of skill in the art to obtain the desired release profiles and timing. In one embodiment, the time release coating is EUDRAGIT RS PO, and the coating will vary in accordance with factors known by those of skill in the art. The amount of time release coat is, in one embodiment, 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 11%, 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; or more, if determined to be appropriate.

[0061] In one embodiment, the controlled release formulation comprising a time delay component does not allow substantial release of the cytidine analog for at least about three hours after oral ingestion by a patient, for at least about four hours after oral ingestion by a patient, for at least about five hours after oral ingestion by a patient, for at least about six hours after ingestion by a patient, for at least about eight hours after ingestion by a patient, or for at least about ten hours after ingestion by a patient. In one embodiment, a dissolution time period is, in one embodiment, between about three hours and about ten hours, between about four hours and about six hours. A nominal lag time of five hours is usually considered sufficient, since small intestinal transit has been considered relatively constant at about 3 to 4 hours. Multiple coatings of either the same or different types of a time delay release component may be used by one of skill in the art to target the large intestine and/or colon.

[0062] Examples of time delay methods are known in the art, and include PULSINCAP device, which consists of a non-disintegrating half capsule shell sealed at the open end with a hydrogel plug. The plug hydrates on contact with gastrointestinal fluid, and swells to an extend that it is expelled from the capsule body, thus releasing the drug. Usually, the time it takes the hydrogel plug to hydrate and eject from the capsule shell defined the lag time prior to drug release, and hence, by altering the composition and size of the hydrogel plug, it is possible to achieve drug release after varying lag times. As another example, a pulsed system, called the TIME CLOCK SYSTEM, comprises a solid dosage form coating with a hydrophobic surfactant layer to which a water-soluble polymer is attached to improve adhesion to the core. The

thickness of the outer layer determines the time required to disperse in an aqueous environment. After dispersion of the outer layer, the core becomes available for dispersion.

[0063] In another embodiment of the present invention, the pharmaceutical compositions may include a bacterially degradable component, such as a coating, in order to delay release of the cytidine analog until reaching the large intestine. For example, the digestive excretions in the human gastrointestinal tract lack specific enzymes that can degrade certain types of oligosaccharides, such as, for example, cellulose. In contrast, bacteria existing at the level of the large intestine have the ability to digest many of these types of polysaccharides. Accordingly, a coating or matrix which undergoes bacterial degradation in the colonic surroundings and dissolves/degrades causing release of its drug content is compatible with the present invention. A number of flora are typically found in the human gastrointestinal tract. The flora may change depending upon the physiological condition of the person or animal being treated. Drug delivery may be designed to specifically target a type of flora known to be in abundance in a patient or a population of patients.

[0064] A partial list of oligosaccharides suitable for incorporation into the controlled release pharmaceutical compositions of the present invention, in one embodiment, includes those which can be digested by colonic bacteria but not by the enzymes present at the level of a patient's stomach or small intestine. Examples of such oligosaccharides are cellobiose, lactulose, the trisaccharide raffinose and stachyose and polymers thereof, such as cellulose. Oligosaccharides also include amylose, arabinogalatan, chitosan, chondroitin, cyclodextrin, dextran, guar gum, inulin, pectin, and xylan. Natural polymers such as mucopolysaccharides can also be the basis of bacterially degradable coatings. Most of these natural polymers are, in their unmodified form, soluble in water and gastric fluid. Accordingly, in one embodiment, natural polymers are cross linked to reduce the hydrophilicity of these polymers and thus allow their utility in the compositions and methods of the invention as colonic drug carriers which pass the small intestine and degrade in the colon. Accordingly, bacterially degradable components are, in one embodiment, covalently or non covalently bonded to a polymer, or mixed with a polymer in one embodiment an acrylic polymer, including, a methacrylic polymer, such as a EUDRAGIT polymer.

[0065] A non-limiting example of one cross-linking method is amide protection by the reaction of diamine with the polymer. Diamines that can be used include: 1,4 butanediamine, 1,6

hexanediamine, 1,7 heptanediamine and 1,12 dodecanediamine. A number of U.S. patents and patent application publications discuss methods by which to control release of drugs via bacterially degradable coatings and/or matrices, such as, for example, U.S. 5,525,634, U.S. 5,849,327, U.S. 4,432,966, U.S. 5,112,621, and U.S. 5,536,507, all of which are incorporated by reference herein in their entireties.

[0066] In one embodiment, the oligosaccharides are formulated together with a seal coat or time release component. In one embodiment, the oligosaccharide is a mixture of chitosan and pectin, in any ratio, for example, 1:1. In another embodiment, the oligosaccharide is amylose. In one embodiment, oligosaccarides and/or mixtures of oligosaccharides are formulated together with EUDRAGIT RS PO. For example, the oligosaccharide or mixture thereof can be present in a bacterially degradable coat in the amount of about 2% w/w of the coat; about 4%, w/w of the coat, about 6%, w/w, of the coat, about 8%, w/w, of the coat; about 10% w/w of the coat; about 12%, w/w of the coat; about 14%, w/w, of the coat, about 16%, w/w/, of the coat; about 18% w/w of the coat; about 20%, w/w of the coat, about 22%, w/w, of the coat, about 24%, w/w, of the coat; about 26% w/w of the coat; about 28%, w/w of the coat, about 30%, w/w, of the coat, about 32%, w/w, of the coat; about 34% w/w of the coat; about 36%, w/w of the coat, about 38%, w/w, of the coat, about 40%, w/w, of the coat; about 42% w/w of the coat; about 44%, w/w of the coat; about 46%, w/w, of the coat, about 48%, w/w/, of the coat; about 50% w/w of the coat; about 52%, w/w of the coat, about 54%, w/w, of the coat, about 56%, w/w, of the coat; about 58% w/w of the coat; about 60%, w/w of the coat, about 62%, w/w, of the coat, about 64%, w/w, of the coat; about 66% w/w of the coat; about 68%, w/w of the coat, about 70%, w/w, of the coat, about 72%, w/w, of the coat; about 74% w/w of the coat; about 76%, w/w of the coat; about 78%, w/w, of the coat, about 80%, w/w/, of the coat; about 82% w/w of the coat; about 84%, w/w of the coat, about 86%, w/w, of the coat, about 88%, w/w, of the coat; about 90% w/w of the coat; about 92%, w/w of the coat, about 91%, w/w, of the coat, about 96%, w/w, of the coat; about 98%, w/w, of the coat, or more, if determined to be appropriate. The balance, in this embodiment, is time release component, for example, EUDRAGIT RS PO, and optionally, plasticizer, each in amounts noted elsewhere herein.

[0067] The presence of microbial anaerobic organisms is known to provide reducing conditions in the large intestine and colon. Thus, the coating may also suitably comprise a material that is redox sensitive. Such coatings typically consist of azo-polymers, which can, for

example, be composed of a random co-polymer of styrene and hydroxyethyl methacrylate cross-linked with divinyl azo-benzene synthesized by free radical polymerization. The azo-polymer is broken down enzymatically and specifically in the colon delivery system. When drugs are coated with these polymers, they are protected against gastric and intestinal enzymes. The drugs are subsequently released in the large intestine where enzyme activity is low and the azo bond is broken by only microbial enzymes in the colon.

[0068] *In vitro* evaluation of azo-containing polysaccharide gels, more specifically azo-inulin and azo-dextran gels, for colonic delivery has been shown that *in vitro* azo-polysaccharide gels can be degraded through both reduction of the azo group in the cross-links as well as enzymatic breakdown of the polysaccharide backbone. The azo-polysaccharide gels were synthesized by radical cross-linking of a mixture of methacrylated inulin or methacrylated dextran and NN-bis-(methacryloylamino) azo-benzene (B(MA)AB), and were characterized by dynamic mechanical analysis and swelling measurements. Azo-dextran gels could be obtained from methacrylated dextran having low degrees of substitution but not from minimally substituted methacrylated inulin. Increasing the amount of B(MA)AB resulted in denser azo-inulin and azo-dextran networks. Compared with their swelling in dimethyl formamide, all azo-dextran gels became more swollen in water while azo-inulin gels shrank upon exposure to water, indicating a more hydrophobic character of the azo-inulin gels. Breakdown of the inulin and dextran chains by inulinase and dextranase, respectively, were observed. In one embodiment, the azo-polymer is a polymer of 2-hydroxyethyl methacrylic acid cross linked with divinyl azobenzene (tradename HEMA-DVAB polymer).

[0069] In one embodiment, the azo polymer is 2-hydroxyethyl methacrylic acid cross linked with divinyl azobenzene (HEMA-DVAB polymer). The amount of azopolymer can be determined by one of skill in the art, and in one embodiment, is present in a bacterially degradable coat in the amount of about 2% w/w of the coat; about 4%, w/w of the coat, about 6%, w/w, of the coat, about 8%, w/w, of the coat; about 10% w/w of the coat; about 12%, w/w of the coat; about 14%, w/w, of the coat, about 16%, w/w/, of the coat; about 18% w/w of the coat; about 20%, w/w of the coat, about 22%, w/w, of the coat, about 24%, w/w, of the coat; about 26% w/w of the coat; about 28%, w/w of the coat, about 30%, w/w, of the coat, about 32%, w/w, of the coat; about 34% w/w of the coat; about 36%, w/w of the coat, about 38%, w/w, of the coat, about 40%, w/w, of the coat; about 42% w/w of the coat; about 44%, w/w of the coat; about

46%, w/w, of the coat, about 48%, w/w/, of the coat; about 50% w/w of the coat; about 52%, w/w of the coat, about 54%, w/w, of the coat, about 56%, w/w, of the coat; about 58% w/w of the coat; about 60%, w/w of the coat, about 62%, w/w, of the coat, about 64%, w/w, of the coat; about 66% w/w of the coat; about 68%, w/w of the coat, about 70%, w/w, of the coat, about 72%, w/w, of the coat; about 74% w/w of the coat; about 76%, w/w of the coat; about 78%, w/w, of the coat, about 80%, w/w/, of the coat; about 82% w/w of the coat; about 84%, w/w of the coat, about 86%, w/w, of the coat, about 88%, w/w, of the coat; about 90% w/w of the coat; about 92%, w/w of the coat, about 91%, w/w, of the coat, about 96%, w/w, of the coat; about 98%, w/w, of the coat, or more, if determined to be appropriate. The balance, in this embodiment, is time release component, for example, EUDRAGIT RS PO, and optionally, plasticizer, each in amounts noted elsewhere herein.

[0070] Below are discussed additional examples of art-known methods of colonic delivery, which incorporate one or more of the methods discussed hereinabove and are suitable for formulating the cytidine compounds. As discussed above, as a general rule, those skilled in the art consider the small intestine to be the preferred target for delivery of acid-labile compounds. Controlled release methods and compositions for targeting or controlling the release of an active compound in the large intestine and colon have been disclosed, including the following. A formulation for site specific release of a cytidine analog in the colon comprises a cytidine analog and amorphous amylose with an outer coating of cellulose or an acrylic polymer material. The active compound is in one embodiment coated with glassy amylose, which tends not to degrade until it reaches the colon where it is attacked by amylose cleaving enzymes provided by microbial flora normally present in the colon. The composition may optionally be further coated with a cellulose or acrylic polymer material, which enhances the delayed release property of the amylose coated composition. The rate of release of the active compound from the composition once it reaches the colon may be controlled by varying the thickness of inner amylose coating provided. It is also possible to vary the release in the colon by coating different particles of the active compound with amylose of different thicknesses. Release characteristics can be further varied by drying, which affects pore size and permeability or by adding a fatty or waxy substance to retard penetration of water. In one embodiment, the cellulose or acrylic polymer outer coating material displays pH independent degradation. Another controlled release composition suitable for delivery of cytidine analog to the colon includes cytidine analog

containing spheres also containing microcrystalline cellulose and having diameters in the range 1.00 to 1.40 mm, which spheres are coated with a mixed solvent (water and an organic water miscible solvent) amylose/ethyl cellulose composition, although the latter may instead be an acrylic polymer or hydrophobic coating. Where higher amylose concentrations are present in the coatings, a thicker coating is applied such that release of the cytidine analog should not take place before the colon. Another sustained release pharmaceutical formulation includes coating particles with different thicknesses of a material (such as cellulose acetophthalate) in order to release the drug compound at different rates so as to provide sustained release over a predetermined time period.

[0071] Generally, methods for sustained release and/or delivery to the large intestine and/or colon include the following methods disclosed in the following U.S. issued patents, all of which are incorporated herein by reference in their entireties. U.S. Pat. No. 5,529,790 discloses pharmaceutical formulations which provide delayed and sustained release of a drug from the formulation by means of a hydratable diffusion barrier coating. The delay is a consequence of the rate of hydration and the thickness of the coating and the sustained release results from the permeability and thickness of the coating. The diffusion barrier, in one embodiment, consists of a film-forming material that is insoluble in intestinal conditions and at least one further additive which controls the rate of hydration and permeability of the diffusion barrier. One embodiment includes an embodiment where film-forming polymers are non-aqueous or aqueous dispersions of fully esterified acrylic resins (e.g. EUDRAGIT NE30D), fully esterified acrylic resins containing quaternary amine side chains (e.g. EUDRAGIT RS30D) or aqueous dispersions of ethyl cellulose. In one embodiment, an additive for controlling the rate of hydration and the permeability is magnesium stearate. The drug (e.g. diltiazem hydrochloride) may be formulated as spherical microparticles having a diameter in the range 500-1500 micrometers and is, in one embodiment, formulated in two batches of particles, a long delay batch having a low rate of hydration and low permeability and a short delay batch having a relatively high rate of hydration and a high permeability, so that sustained release of the drug can be effected over an extended period of time. Dissolution studies were carried out on particles having different coating thicknesses. U.S. Pat. No. 5,260,069 and U.S. Pat. No. 5,834,024 disclose pharmaceutical compositions comprising at least two pluralities of particles. The pluralities may be coated with different thicknesses of a coating material comprising a polymer blend. The blend comprises, as

a major component, at least one water insoluble polymer and, as a minor component, a polymer whose solubility is dependent on pH. U.S. Pat. No. 5,260,069 exemplifies compositions in which nifedipine and zidovudine are active components and U.S. Pat. No. 5,834,024 exemplifies the use of diltiazem as the active component. U.S. Pat. No. 6,267,990 discloses a pharmaceutical composition comprising three pluralities of particles, one of which is uncoated and the other two are coated with different thicknesses of a pH dependent release coating material. U.S. Pat. No. 6,267,990 exemplifies the use of the ACE inhibitor, captopril, as the active component. U.S. Pat. No. 5,834,021 exemplifies a pharmaceutical composition comprising a plurality of pellets comprising prednisolone metasulphobenzoate. The pellets are coated with a first pH dependent release coating material and then filled into a capsule which is then itself coated with a second pH dependent release coating material.

[0072] The drug release controlling component may also optionally include a plasticizer. The plasticizer can be added to coating solutions and dispersions to improve the mechanical properties of the polymeric film in the dry state and to influence permeability and drug release when the product is in contact with the release media. Plasticizers can also induce and enhance the coalescence of the colloidal polymer particles in a homogeneous film by reducing the glass transition and minimum film formation temperature (MFT) and to improve the mechanical properties of the dried films. In one embodiment, the plasticizer, by allowing the coating to "flex" slightly, helps to prevent any premature release of the cytidine analog. In one embodiment, the plasticizer is a water-soluble plasticizers, such as, for example, triethyl citrate, triacetin; in another embodiment, the plasticizer is a water-insoluble plasticizers, such as, for example, tributyl citrate, acetyltributyl citrate, acetyltriethylcitrate, dibutyl sebacate, dibutyl phthalate and diethyl phthalate. The plasticizer, if added, may be present at any level known to those of skill in the art to be effective. In some embodiments, the plasticizer is present in the drug release controlling component in an amount of about 2%, about 3%, about 4%, about 5%, about 6%, about 7%, about 8%, about 9%, about 10%, about 11%, about 12%, about 14%, about 16%, about 18%, about 20%, about 25%, w/w, of the coat, or more. In some embodiments, the plasticizer is present in an amount of between about 4% and about 12%, between about 5% and about 11%, between about 6% and about 9%; between about 5% and 40%, between about 5% and 30%, between about 5% and about 20%, w/w, of the coat..

[0073] In addition to the cytidine and drug release controlling component, pharmaceutical compositions of the present invention will, in one embodiment, contain one ore more other excipients to form a drug "core". These excipients include diluents (bulking agents), lubricants, disintegrants, fillers, stabilizers, surfactants, preservatives, coloring agents, flavoring agents, binding agents, excipient supports, glidants, permeability enhancement excipients, plasticizers and the like, all of which are known in the art; all named excipients are optional components. 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 disintegrants that help break the tablet apart once it reaches the target delivery site. Selection of excipients and the amounts to use may be readily determined by the formulation scientist based upon experience and consideration of standard procedures and reference works in the field.

[0074] Binders are used to impart cohesive qualities to a tablet, and thus ensure that the tablet remains intact after compression. Suitable binder materials 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 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 t; 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 91%, 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; or between about 5% and about

[0075] Diluents are typically necessary 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 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. Generally, diluents are used in amounts calculated to obtain a volume tablet or capsule that is desired; in some embodiments, a diluent is used in an amount of about 5% or more, about 10% or more, about 15% or more, 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, 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.

[0076] Lubricants are used 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. Stearates, if present, in one embodiment represent at no more than approximately 2 wt. % of the drug-containing core. Further examples of lubricants include 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 one embodiment, the binding agent is magnesium stearate, and is present, relative to the drug core, in the 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; between about 1% and about 3% w/w of the drug core.

[0077] Disintegrants are used to facilitate disintegration of the tablet, and are generally starches, clays, celluloses, algins, gums or crosslinked polymers. Disintegrants also include 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 alginic acid, calcium alginate, calcium carboxymethylcellulose, chitosan, colloidal silicon dioxide, sodium croscarmellose, crospovidone, sodium docusate, guar gum, hydroxypropyl cellulose, magnesium aluminum silicate, methylcellulose, microcrystalline cellulose, potassium polacrilin, povidone, powdered cellulose, sodium alginate, sodium carboxymethyl cellulose, sodium starch glycolate, starch, and pregelatinized starch. The disintegrant can be, relative to the drug core, 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 t; 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; between about 4% and about 6% w/w of the drug core.

[0078] Stabilizers (also called absorption ehancers) are used to inhibit or retard drug decomposition reactions that include, by way of example, oxidative reactions. Stabilizing agents include 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. The stabilizer can be, relative to the drug core, 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 t; 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; between about 4% and about 6% w/w of the drug core.

[0079] Glidants can be added to improve the flow properties of a powder composition or granulate and improve the accuracy of dosing. Excipients that may function as glidants include colloidal silicon dioxide, magnesium trisilicate, powdered cellulose, starch, tribasic calcium phosphate, alcium silicate, powdered cellulose, colloidal silicon dioxide, magenesium silicate, magnesium trisilicate, silicon dioxide, starch, tribasic calcium phosphate, and talc. Appropriate amounts to use may be determined by those of skill in the art.

[0080] Permeation enhancers are an included excipient in one embodiment. Permeation enhancers act to enhance uptake of a substance through the intestinal wall and deliver more of a substance to the bloodstream. Movement through the intestinal wall may occur by passive diffusion, the movement of drug across a membrane in a manner driven solely by the concentration gradient; by carrier-mediated diffusion, movement of drug across a cell membrane

via a specialized transport system embedded in the cell membrane; paracellular diffusion, the movement of drug across a membrane by going between, rather than through, two cells; and transcellular diffusion, the movement of a drug across the cell. Additionally, there are numerous cellular proteins capable of preventing intracellular accumulation of drugs by pumping drug that enters the cell back out. These are sometimes called efflux pumps. One of the most important is p-glycoprotein, which is present in many different tissues in the body (e.g., intestine, placental membrane, blood-brain barrier). Permeation enhancers can work by facilitating any of the processes mentioned above (such as by increasing fluidity of membranes, opening "tight junctions" between cells, and/or inhibiting efflux.)

[0081] Examples of suitable permeation inhibitors include, for example, but are not limited to, surfactants. Suitable examples for the present invention include are known and commercially available, e.g. from the BASF company under the trade mark SOLUTOL. An example is SOLUTOL HS15 which is known, e.g. from the BASF technical leaflet MEF 151E (1986), to comprise of about 70% polyethoxylated 12-hydroxystearate by weight and about 30% by weight unesterified polyethylene glycol component. SOLUTOL HS 15 has a hydrogenation value of 90 to 110, a saponification value of 53 to 63, an acid number of maximum 1, and a maximum water content of 0.5% by weight. Polyoxyethylene-polyoxypropylene co-polymers and block co-polymers are included in one embodiment, for example of the type known and commercially available under the trade names PLURONIC, EMKALYX and POLOXAMER. A further example of this class is POLOXAMER F127. Propylene glycol mono- and di-fatty acid esters such as propylene glycol dicaprylate (also known and commercially available under the trade name MIGLYOL 840), propylene glycol dilaurate, propylene glycol hydroxystearate, propylene glycol isostearate, propylene glycol laurate, propylene glycol ricinoleate, propylene glycol stearate and so forth are also included in some embodiments. Other examples include propylene glycol mono C:8 esters include SEFSOL 218 (Nikko Chemicals) and CAPRYOL 90 (Gattefosse) and tocopherol esters, e.g. tocopheryl acetate and tocopheryl acid succinate (HLB of about 16), transesterified ethoxylated vegetable oils are known and are commercially available under the trade name LABRAFIL. Examples are LABRAFIL M 2125 CS (obtained from corn oil and having an acid number of less than about 2, a saponification number of 155 to 175, an HLB value of 3 to 4, and an iodine number of 90 to 110), and LABRAFIL M 1944 CS (obtained from kernel oil and having an acid number of about 2, a saponification number of 145 to 175 and

an iodine number of 60 to 90). LABRAFIL M 2130 CS (which is a transesterification product of a C.sub.12-18 glyceride and polyethylene glycol and which has a melting point of about 35 to 40.degree. C., an acid number of less than about 2, a saponification number of 185 to 200 and an iodine number of less than about 3). In one embodiment, the transesterified ethoxylated vegetable oil is LABRAFIL M 2125 CS which can be obtained, for example, from Gattefosse, Saint-Priest Cedex, France. In one embodiment, a permeation enhancer includes water soluble tocopheryl polyethylene glycol succinic acid esters (TPGS), e.g. with a polymerisation number ca 1000, e.g. available from Eastman Fine Chemicals Kingsport, Tenn., USA. Other embodiments include POLOXAMER compounds, particularly F127, chitosan, carboxymethylcellulose, SOLUTOL compounds, sodium laurate, and LABRAFIL compounds. Other permeation enhancers include 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. Appropriate amounts to use can be determined by one of skill in the art.

[0082] In some embodiments, the present invention comprises a controlled release pharmaceutical composition for oral administration for enhanced systemic delivery of a cytidine analog comprising a therapeutically effective amount of a cytidine analog and a drug release controlling component which is capable of providing release of the cytidine analog primarily in the large intestine. The present invention in some embodiments includes a controlled release pharmaceutical composition for oral administration for enhanced systemic delivery of 5-azacytidine consisting essentially of or consisting of a therapeutically effective amount of 5-azacytidine and a drug release controlling component which is capable of providing release of the cytidine analog primarily in the large intestine. In other embodiments, the present invention includes a controlled release pharmaceutical composition for oral administration of a cytidine analog for enhanced systemic delivery of the cytidine analog consisting essentially or consisting of a therapeutically effective amount of a cytidine analog and an enteric coating which is capable of providing release of the cytidine analog primarily in the large intestine. In other embodiments, the present invention includes a controlled release pharmaceutical composition for oral administration for enhanced systemic delivery of a cytidine analog consisting essentially of (or consisting of) a therapeutically effective amount of 5-azacytidine and an enteric coating which is capable of providing release of the cytidine analog primarily in the large intestine and at

least one excipient which improves the cohesive qualities of the, and/or increases the bulk of, and/or improves the manufacture of, or facilitates disintegration of, and/or retards the drug decomposition reactions occurring in, or enhances uptake through the intestinal wall of, the controlled release pharmaceutical compositions of the present invention.

[0083] A tablet can be made by compressing a powder composition granulate between a punch and dye. Some excipients and active ingredients have a tendency to adhere to the surfaces of the punch and dye, which can cause the tablet to have pitting and other surface irregularities. A lubricant may be added to the composition to reduce adhesion and ease release of the product form the dye. Lubricants include magnesium stearate, calcium stearate, glyceryl monostearate, glyceryl palmitostearate, hydrogenated castor oil, hydrogenated vegetable oil, mineral oil, polyethylene glycol, sodium benzoate, sodium lauryl sulfate, sodium stearyl fumarate, stearic acid, talc and zinc stearate.

[0084] Suitable patients to treat include humans; birds such as chickens, ostriches, quail, and turkeys; mammals such as companion animals (including dogs, cats, and rodents) and economic food and/or fur or other product animals, such as horses, cattle, llamas, chinchillas, ferrets, goats, sheep, rodents, minks, rabbits, raccoons, and swine.

[0085] In another embodiment, the present invention includes a method for delivering a cytidine analog comprising administering to a patient in need thereof a composition of the present invention. In one embodiment, the composition comprises an oral formulation of a cytidine analog, wherein the oral formulation of the cytidine analog comprises a) a therapeutically effective amount of a cytidine analog and b) a drug release controlling component capable of providing release of the cytidine analog primarily in the large intestine, wherein after ingestion by a patient the cytidine analog is released primarily in the large intestine.

[0086] Another embodiment of the present invention includes a method of formulating a cytidine analog for oral delivery, comprising formulating (in one embodiment, coating) a therapeutically effective amount of a cytidine analog with a drug release controlling component capable of providing release of the cytidine analog primarily in the large intestine using methods disclosed in the present disclosure.

[0087] In another embodiment, the present invention includes a method of increasing the bioavailability of a cytidine analog comprising administering the controlled release

pharmaceutical compositions of the present invention to a patient. Specifically, a controlled release pharmaceutical composition of the present invention is provided to a patient, and ingested by the patient, where the composition contacts the biological fluids of the patient's body and increases the bioavailability of the cytidine analog. Oral bioavailability of a cytidine analog in the compositions of the present invention can be more than 5%, more than 10%, more than 15%, more than 20%, more than 25%, more than 30% or more than 50% greater than the oral bioavailability of prior art formulations of a cytidine analog. Average maximum plasma concentration achieved relative to the dose administered may be more than 2 fold higher, 3 fold higher, 5 fold higher, about 10 fold higher than the oral bioavailability of prior art formulations of a cytidine analog when a cytidine analog is administered orally in the controlled release formulations of the present invention.

[0088] In another embodiment, the present invention includes methods for treating a patient having a disease associated with abnormal cell proliferation, comprising administering the controlled release pharmaceutical compositions of the present invention. In one embodiment, the controlled release pharmaceutical compositions of the present invention allow for enhanced bioavailablity of the cytidine analog to the patient.

[0089] In some embodiments, indications that may be treated using the pharmaceutical compositions of the present invention include those involving undesirable or uncontrolled cell proliferations. Such indications include 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.

[0090] Generally, cells in a benign tumor retain their differentiated features and do not divide in a completely uncontrolled manner. A benign tumor is usually localized and nonmetastatic. Specific types of benign tumors that can be treated using the present invention include hemangiomas, hepatocellular adenoma, cavernous haemangioma, 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.

[0091] In a malignant tumor cells that become undifferentiated, do not respond to the body's growth control signals, and multiply in an uncontrolled manner. The malignant tumor is invasive and capable of spreading to distant sites (metastasizing). Malignant tumors are generally 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.)

[0092] Specific types of cancers or malignant tumors, either primary or secondary, that can be treated using this invention include leukemia, breast cancer, skin cancer, bone cancer, prostate cancer, liver cancer, 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, small-cell lung 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.

[0093] 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.

[0094] 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.

[0095] 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.

[0096] 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.

[0097] 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.

[0098] 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. In one embodiment, MDS is a condition to treat with the present invention, and includes 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.

[0099] Treatment of abnormal cell proliferation due to insults to body tissue during surgery may be possible for a variety of surgical procedures, including joint surgery, bowel surgery, and cheloid scarring. Diseases that produce fibrotic tissue include emphysema.

Repetitive motion disorders that may be treated using the present invention include carpal tunnel syndrome. An example of cell proliferative disorders that may be treated using the invention is a bone tumor.

[00100] The proliferative responses associated with organ transplantation that may be treated using this invention include those proliferative responses contributing to potential organ rejections or associated complications. Specifically, these proliferative responses may occur during transplantation of the heart, lung, liver, kidney, and other body organs or organ systems.

[00101] Abnormal angiogenesis that may be may be treated using this invention include those abnormal angiogenesis accompanying rheumatoid arthritis, ischemic-reperfusion related brain edema and injury, cortical ischemia, ovarian hyperplasia and hypervascularity, (polycystic ovary syndrom), endometriosis, psoriasis, diabetic retinopaphy, and other ocular angiogenic diseases such as retinopathy of prematurity (retrolental fibroplastic), macular degeneration, corneal graft rejection, neuroscular glaucoma and Oster Webber syndrome.

[00102] Diseases associated with abnormal angiogenesis require or induce vascular growth. For example, corneal angiogenesis involves three phases: a pre-vascular latent period, active neovascularization, and vascular maturation and regression. The identity and mechanim of various angiogenic factors, including elements of the inflammatory response, such as leukocytes, platelets, cytokines, and eicosanoids, or unidentified plasma constituents have yet to be revealed. The pharmaceutical composition of the present invention may also be used for treating diseases associated with undesired or abnormal angiogenesis alone or in conjunction with an anti-angiogenesis agent.

[00103] The particular dosage of these agents required to inhibit angiogenesis and/or angiogenic diseases may depend on the severity of the condition, the route of administration, and related factors that can be decided by the attending physician. Generally, accepted and effective daily doses are the amount sufficient to effectively inhibit angiogenesis and/or angiogenic diseases. According to this embodiment, the pharmaceutical composition of the present invention may be used to treat a variety of diseases associated with undesirable angiogenesis such as retinal/choroidal neuvascularization and corneal neovascularization. Examples of retinal/choroidal neuvascularization include, but are not limited to, Bests diseases, myopia, optic pits, Stargarts diseases, Pagets disease, vein occlusion, artery occlusion, sickle cell anemia, sarcoid, syphilis, pseudoxanthoma elasticum carotid abostructive diseases, chronic

uveitis/vitritis, mycobacterial infections, Lyme's disease, systemic lupus erythematosis, retinopathy of prematurity, Eales disease, diabetic retinopathy, macular degeneration, Bechets diseases, infections causing a retinitis or chroiditis, presumed ocular histoplasmosis, pars planitis, chronic retinal detachment, hyperviscosity syndromes, toxoplasmosis, trauma and post-laser complications, diseases associated with rubesis (neovascularization of the angle) and diseases caused by the abnormal proliferation of fibrovascular or fibrous tissue including all forms of proliferative vitreoretinopathy. Examples of comeal neuvascularization include, but are not limited to, epidemic keratoconjunctivitis, Vitamin A deficiency, contact lens overwear, atopic keratitis, superior limbic keratitis, pterygium keratitis sicca, sjogrens, acne rosacea, phylectenulosis, diabetic retinopathy, retinopathy of prematurity, corneal graft rejection, Mooren ulcer, Terrien's marginal degeneration, marginal keratolysis, polyarteritis, Wegener sarcoidosis, Scleritis, periphigoid radial keratotomy, neovascular glaucoma and retrolental fibroplasia, syphilis, Mycobacteria infections, lipid degeneration, chemical burns, bacterial ulcers, fungal ulcers, Herpes simplex infections, Herpes zoster infections, protozoan infections and Kaposi sarcoma.

[00104] The pharmaceutical composition of the present invention may be used for treating chronic inflammatory diseases associated with abnormal angiogenesis. The chronic inflammation depends on continuous formation of capillary sprouts to maintain an influx of inflammatory cells. The influx and presence of the inflammatory cells produce granulomas and thus, maintains the chronic inflammatory state. Inhibition of angiogenesis using the composition of the present invention may prevent the formation of the granulosmas, thereby alleviating the disease. Examples of chronic inflammatory disease include, but are not limited to, inflammatory bowel diseases such as Crohn's disease and ulcerative colitis, psoriasis, sarcoidois, and rhematoid arthritis.

[00105] Inflammatory bowel diseases such as Crohn's disease and ulcerative colitis are characterized by chronic inflammation and angiogenesis at various sites in the gastrointestinal tract. For example, Crohn's disease occurs as a chronic transmural inflammatory disease that most commonly affects the distal ileum and colon but may also occur in any part of the gastrointestinal tract from the mouth to the anus and perianal area. Patients with Crohn's disease generally have chronic diarrhea associated with abdominal pain, fever, anorexia, weight loss and abdominal swelling. Ulcerative colitis is also a chronic, nonspecific, inflammatory and ulcerative

disease arising in the colonic mucosa and is characterized by the presence of bloody diarrhea. These inflammatory bowel diseases are generally caused by chronic granulomatous inflammation throughout the gastrointestinal tract, involving new capillary sprouts surrounded by a cylinder of inflammatory cells. Inhibition of angiogenesis by the composition of the present invention should inhibit the formation of the sprouts and prevent the formation of granulomas. The inflammatory bowel diseases also exhibit extra intestinal manifestations, such as skin lesions. Such lesions are characterized by inflammation and angiogenesis and can occur at many sites other the gastrointestinal tract. Inhibition of angiogenesis by the composition of the present invention should reduce the influx of inflammatory cells and prevent the lesion formation.

[00106] Sarcoidois, another chronic inflammatory disease, is characterized as a multisystem granulomatous disorder. The granulomas of this disease can form anywhere in the body and, thus, the symptoms depend on the site of the granulomas and whether the disease is active. The granulomas are created by the angiogenic capillary sprouts providing a constant supply of inflammatory cells. By using the composition of the present invention to inhibit angionesis, such granulomas formation can be inhibited. Psoriasis, also a chronic and recurrent inflammatory disease, is characterized by papules and plaques of various sizes. Treatment using the composition of the present invention should prevent the formation of new blood vessels necessary to maintain the characteristic lesions and provide the patient relief from the symptoms.

[00107] Rheumatoid arthritis (RA) is also a chronic inflammatory disease characterized by non-specific inflammation of the peripheral joints. It is believed that the blood vessels in the synovial lining of the joints undergo angiogenesis. In addition to forming new vascular networks, the endothelial cells release factors and reactive oxygen species that lead to pannus growth and cartilage destruction. The factors involved in angiogenesis may actively contribute to, and help maintain, the chronically inflamed state of rheumatoid arthritis. Treatment using the composition of the present invention alone or in conjunction with other anti-RA agents should prevent the formation of new blood vessels necessary to maintain the chronic inflammation and provide the RA patient relief from the symptoms.

[00108] The pharmaceutical composition of the present invention may also be used to treat autoimmune diseases. Autoimmune diseases refer to a wide range of degenerative diseases caused by the immune system attacking a person's own cells. Autoimmune diseases are usually classified clinically in a variety of ways. In light of affected parts by the diseases, there are, for

example, degenerative diseases of supporting tissues and connective tissues; autoimmune degenerative diseases of salivary glands, particularly Sjogren's disease; autoimmune degenerative diseases of kidneys, particularly systemic lupus erythematodes (SLE) and glomerulonephritis; autoimmune degenerative diseases of joints, particularly rheumatoid arthritis; and autoimmune degenerative diseases of blood vessels such as generalized necrotizing angitis and granulomatous angitis; and multiple sclerosis. Alternatively, autoimmune diseases can be classified in one of the two different categories: cell-mediated disease (i.e. T-cell) or antibody mediated disorders. Examples of cell-mediated autoimmune diseases include multiple sclerosis, rheumatoid arthritis, autoimmune thyroiditis, and diabetes mellitus. Antibody-mediated autoimmune disorders include myasthenia gravis and SLE.

[00109] Dosing schedules for the compositions and methods of the present invention, 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. Appropriate biomarkers may be used to evaluate the drug's effects on the disease state and provide guidance to the dosing schedule. In some cases, 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 of the present invention including the daily administration to a patient in need thereof of. 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.

[00110] The following examples are provided for illustrative purposes only and are not intended to limit the scope of the invention.

EXAMPLES

Example 1

[00111] Absorption Potential Assessment of 5-azacytidine using Caco 2 Monolayers

[00112] The permeability of 5-azacytidine was determined in a Caco 2 monolayer model system using phosphate buffered saline as the system medium. The Caco-2 cells are an intestinal epithelial cell line (human colon adenocarcinoma established from the primary colon tumor (adenocarcinoma)) Monolayers of Caco-2 cells are used to classify the intestinal absorption potential of a drug candidate molecule. The assay was carried out in accordance with P.

Artursson and J. Karlsson, "Correlation between Oral Drug Absorption in Humans and Apparent Drug Permeability Coefficients in Human Intestinal Epithelial (Caco-2) Cells", *Biochem. Biophys. Res. Commun.* 175, 880 (1991).

[00113] Briefly, Caco-2 cells were grown to confluence on collagen-coated, microporous polycarbonate in 12-well plates. For the assay buffer, Dulbecco's Phosphate Buffered Saline at pH 7.4 was used. The chamber on the apical side of the cells was filled with DPBS containing 1000 micromolar 5-azacytidine with or without additional excipients. The test compound was then dosed on either the apical or basolateral side of the Caco-2 monolayer, and flux across the monolayer was determined both one and two hours after dosing. Results were compared to control high permeability compounds metoprolol and antipyrine and low permeability compounds atenolol and ranitidine, and were expressed as Papp (apparent permeability) compared to reference compound standards. Integrity of the monolayers was determine both before and after testing by measuring the transendothelial electrical resistance (TEER) value. Apparent permeability is calculated as $(dC_r/dt) \times V_r/(A \times C_0)$ where dC_r/dt is the slope of the cumulative concentration in the receiver compartment versus time in micromolar per second, V_r is the volume of the receiver compartment in cubic centimeters, A is the area of the cell monolayer, and C_0 is the measured initial donor concentration in micromolar.

[00114] In the absence of additional excipients the measured apical to basolateral 5-azacytidine permeability for 5-azacytidine was $0.15 \pm 0.02 \times 10^{-6}$ cm/sec.

[00115] Additionally, a series of pharmaceutically acceptable excipients were screened for their ability to increase the apparent permeability of 5-azacytidine in this model system. The excipients evaluated and their effects on 5-azacytidine permeability in this model system are presented in Table 1.

[00116] Table 1. Sodium laurate was obtained from Sigma Chemical (available from St. Louis, MO); Vitamin E TPGS (TPGS-TPGS –d-alpha tocopherol polyethylene glycol 1000 succinate (available from Eastman, Kingsport, TN); LABRAFIL M 1944 CS(2) (Oleyl macrogolglycerides) (available from Gattefosse, France).

[00117] **Table 1: Effect of 5-azacytidine Permeability in Caco 2 Monolayer Model with Various Excipients**

Excipient	Excipient Concentration (%)	P_{app} ($x10^{-6}$ cm/sec)
Control	NA	0.15 ± 0.02
Poloxamer F127	0.1	0.13 ± 0.02
Chitosan	0.1	0.15 ± 0.01
Carboxymethyl Cellulose	0.1	0.26 ± 0.09
Solutol	0.1	0.26 ± 0.04
Sodium Laurate	0.1	15.77 ± 1.77*
	0.05	0.17 ± 0.03
	0.01	0.19 ± 0.02
	0.001	0.19 ± 0.04
Labrafil	0.1	0.34 ± 0.002
	0.05	0.38 ± 0.13
	0.01	0.23 ± 0.04
	0.001	0.27 ± 0.02
TPGS	0.1	0.38 ± 0.09
	0.05	0.45 ± 0.18
	0.01	0.30 ± 0.05
	0.001	0.33 ± 0.08

* High permeability due to toxic effects of sodium laurate on Caco 2 cells

[00118] Conclusion

[00119] 5-azacytidine was found to permeate through Caco-2 monolayers, indicating that colonic epithelial cells are a good candidate for delivery of 5-azacytidine for enhanced bioavailability. Permeation of 5-azacytidine was found to be enhanced by TPGS and Labrafil, having a significant effect with respect to increased 5-azacytidine permeability. For both excipients, there appeared to be a shallow dose response relationship between the amount of excipient and the observed permeability. Inclusion of appropriate amounts of TPGS and/or Labrafil results in an increase in the apparent permeability of 5-azacytidine without adverse effects on Caco 2 monolayers. Inclusion of one or both of these excipients should improve oral bioavailability of 5-azacytidine through enhanced GI absorption.

Example 2

[00120] Permeability in Human Intestinal Strips

[00121] The permeability of 5-azacytidine has also been assessed in viable human intestinal strips derived from specific sections of the GI tract. This model allows evaluation of

the absorption potential of drugs and differences in drug absorption across the human jejunum, ileum, and colon and is based on Ungell et al. "Membrane Transport of Drugs in Different Regions of the Intestinal Tract of the Rat", *J. Pharm. Sci.* 87:360-366, (1998) and Nejdfors et al. "Mucosal *in vitro* Permeability in the Intestinal Tract of the Pig, the Rat, and Man: Species and Region Related Differences", *Scand. J. Gastroenterol.* 35:501-507, (2000). Permeation experiments were performed for 5-azacytidine in the jejunal, ileal, and colonic tissues originating from the same human donor. Post-mortem human whole intestine was obtained from the International Institute for the Advancement of Medicine (IIAM). Tissues were used within 24 hours of the organ's removal. Tissues were maintained in cold transport media before being stripped. The segments were cut along the mesenteric border, the underlying musculature was stripped off, and the epithelium was rinsed with ice cold normal saline. The stripped tissues were mounted within the vertical USSING chambers (Harvard Apparatus, Holliston, MA). In these studies, 5-azacytidine permeability was measured in both absolute rate and relative to atenolol (internal control). In all cases the net apparent permeability was assessed in the mucosal to serosal direction only. Evaluation conditions included the use of 5-azacytidine alone and for some samples 5-azacytidine in the presence of ketoconazole and THU as enzymatic inhibitors of CYP3A4 and cytidine deaminase, respectively.

[00122] Absolute permeability values for multiple donors at different intestinal sites with and without enzymatic inhibition are presented in graphical form in FIG. 1. Figure 1 shows absolute mucosal to serosal permeability of 5-azacytidine in human intestinal tissue with and without enzymatic inhibition. In general permeability appeared greatest in the colon relative to the jejunum and ileum. Inclusion of enzymatic inhibitors increased the absolute permeability in all GI tract regions, however the size of the increase was maximal in the jejunum and ileum and was relatively small in the colon.

[00123] The permeability of 5-azacytidine in the same human intestinal strips relative to the internal control (atenolol) is shown graphically in FIG. 2. Figure 2 shows relative mucosal to serosal permeability of 5-azacytidine in human intestinal tissue with and without enzymatic inhibition with respect to atenolol. The use of internal controls ensures that small variations due to tissue viability and processing are normalized. Qualitatively, the same conclusions can be drawn from the 5-azacytidine permeability data relative to atenolol. Absorption was greatest and

most consistent between donors in colonic tissue, and the effects of enzymatic inhibition were minimized in tissues derived from the colon.

[00124] Permeation Enhancement in Human Tissues

[00125] Evaluation of the effects of 5-azacytidine permeability in human colonic strips with various levels of the permeation enhancers identified in the Caco 2 model system has also been performed. Absolute and relative permeability of 5-azacytidine with the two excipients at various levels are presented in FIGS. 3 and 4, respectively. Figure 3 shows absolute mucosal to serosal permeability of 5-azacytidine in human colonic tissue with various concentrations of TPGS or LABRAFIL without enzymatic inhibition, and Figure 4 shows relative mucosal to serosal permeability of 5-azacytidine in human colonic tissue with various concentrations of TPGS or LABRAFIL without enzymatic inhibition.

[00126] There was a trend toward greater permeability with increasing levels of TPGS, albeit to a lesser extent than observed in the model Caco2 system. Conversely, under the same conditions Labrafil did not appear to affect 5-azacytidine permeability in human colonic tissue.

[00127] Conclusions

[00128] The available data from viable human intestinal tissue shows that enzymatic degradation of 5-azacytidine was more prevalent in upper GI segments. Effective administration of 5-azacytidine with delivery to the jejunum and/or ileum may require the use of an enzymatic inhibitor such as THU. By contrast, both the absolute and relative permeability of 5-azacytidine appeared maximal in human colonic tissue. Furthermore, enzymatic inhibition in colonic tissue did not provide a dramatic improvement in 5-azacytidine permeability. Therefore, an oral 5-azacytidine dosage form that targets the colon for delivery has been shown to maximize bioavailability without the need to include an enzymatic inhibitor.

Example 3

[00129] Solid Oral Dosage Form

[00130] Solid oral dosage forms of 5-azacytidine were prepared using standard pharmaceutical excipients and techniques. TPGS was first adsorbed onto either microcrystalline cellulose or calcium silicate in an independent step. Dry ingredients were then dry blended and tablets prepared by direct compression. Tablets were then enteric coated with EUDRAGIT S100

from an acetone - isopropanol solvent mixture or with AQUAT AS-HG from a methylene chloride – ethanol solvent mixture.

[00131] Clinical Trial Material cores were composed of the following materials in these ratios:

Ingredient	mg/tablet	% w/w
5-azacytidine	20.0	20.0
Mannitol, USP	58.2	58.2
Microcrystalline Cellulose, NF	15.0	15.0
Crospovidone, NF	3.0	3.0
Magnesium Stearate, NF	1.8	1.8
Vitamin E TPGS, NF	2.0	2.0

[00132] Cores were then be coated to approximately 7% w/w with the following mixture:

Ingredient	mg/tablet	% w/w
EUDRAGIT S100, NF	5.0	71.5
Triethyl citrate, NF	0.5	7.0
Talc, USP	1.5	21.5

[00133] Excipient compatibility studies have demonstrated that 5-azacytidine is compatible with each excipient. Stability studies have demonstrated excellent stability of both cores and coated tablets under long term (25°C, 60% RH) and accelerated (40°, 70% relative humidity) storage conditions. The formulation composition that has been manufactured is presented in Table 2.

[00134] **Table 2: Oral 5-azacytidine Tablet Composition**

Material	Trade Name	Purpose	Quality Standard
5-azacytidine	NA	Active	In-House
Mannitol	PARTECK M200	Bulking Agent	USP
Microcrystalline Cellulose	PROSOLV 90HD	Binding Agent	USP
Crospovidone	POLYPLASONE XL	Disintegrant	USP
Magnesium Stearate	NA	Lubricant	USP
Vitamin E TPGS	NA	Absorption Enhancement	NF

Methacrylic acid copolymer[1]	EUDRAGIT S100[2]	Enteric Coating	USP
Triethyl Citrate	MORFLEX	Plasticizer	USP
Talc,		Anticaking Agent	USP

[1] Hypromellose Acetate Succinate, NF alternate material
[2] AQUAT AS-HG, alternate trade name

Example 4

[00135] Solid Oral Dosage Form

[00136] Solid oral dosage forms of 5-azacytidine were prepared using standard pharmaceutical excipients and techniques. TPGS was first adsorbed onto either microcrystalline cellulose or calcium silicate in an independent step. Dry ingredients were then dry blended and tablets prepared by direct compression. Tablets were then film coated with Klucel EF from ethanol followed by enteric coated with EUDRAGIT S100 from an acetone - isopropanol solvent mixture.

[00137] Clinical Trial Material cores were composed of the following materials in these ratios:

Ingredient	**mg/tablet**	**% w/w**
5-azacytidine	20.0	20.0
Mannitol, USP	43.2	43.2
Microcrystalline Cellulose, NF	30.0	30.0
Crospovidone, NF	3.0	3.0
Magnesium Stearate, NF	1.8	1.8
Vitamin E TPGS, NF	2.0	2.0

[00138] Cores were then sub-coated to approximately 4% w/w with the following materials in ethanol:

Ingredient	**mg/tablet**	**% w/w**
Klucel EF, NF	4.0	6.0

[00139] Film coated cores were then be coated to approximately 7% w/w with the following mixture:

Ingredient	**mg/tablet**	**% w/w**
Eudragit S100, NF	6.0	86.3
Triethyl citrate, NF	1.0	13.7

[00140] Excipient compatibility studies have demonstrated that 5-azacytidine is compatible with each excipient. Stability studies have demonstrated excellent stability of coated tablets under long term (25°C, 60% RH) and accelerated (40°, 70% RH) storage conditions. An additional formulation composition that has been manufactured is presented in Table 3.

[00141] **Table 3: Oral 5-azacytidine Tablet Composition**

Material	Trade Name	Purpose	Quality Standard
5-azacytidine	NA	Active	In-House
Mannitol	Parteck M200	Bulking Agent	USP
Microcrystalline Cellulose	Prosolv 90HD	Binding Agent	USP
Crospovidone	Polyplasone XL	Disintegrant	USP
Magnesium Stearate	NA	Lubricant	USP
Vitamin E TPGS	NA	Absorption Enhancement	NF
Hydroxypropyl Cellulose	Klucel EF	Sub-Coating	NF
Methacrylic acid copolymer[1]	Eudragit S100[2]	Enteric Coating	USP
Triethyl Citrate	Morflex	Plasticizer	USP

[1] Hypromellose Acetate Succinate, NF alternate material
[2] Aquat AS-HG, alternate trade name

Example 5

[00142] A clinical study using the oral formulation described in Example 3 was performed to assess the safety and bioavailability of single oral doses of 5-azacytidine in patients with myelodysplastic syndromes, acute myelogenous leukemia, or solid tumors. The study was a multicenter, open-label, single treatment study. Patients were treated with escalating doses, in 20 mg increments, up to 200 mg. The study assessed the safety and tolerability of escalating doses, provided pilot information on the oral bioavailability of the study drug, and provided information on the single dose pharmacokinetics of the study drug after oral administration.

[00143] Study Design

[00144] Multicenter, open-label, single-treatment, escalating-dose PK study. 1 subject was to receive an oral dose of 5-azacytidine 60 mg (three 20 mg tablets). Single subject cohorts were used for each dose escalation. Subsequent subjects were to be treated at escalating doses, up to 200 mg, in 20 mg increments. Dose escalation was to continue until 1 of the following conditions was reached:

a. Drug was deemed intolerable (i.e., if a subject experienced any Grade 3 or 4 adverse event [AE] possibly related to 5-azacytidine, or the investigator identified any safety concern following drug treatment); or
b. Appropriate concentrations were achieved (defined as ≥4 consecutive timed plasma samples containing quantifiable 5-azacytidine concentrations amenable to PK assessments); or
c. Dose escalation reached the 200 mg level, which is approximately equivalent to the maximum approved daily SC dose of 5-azacytidine (i.e., 100 mg/m^2).

[00145] If drug was deemed intolerable, a 2^{nd}, and then a 3^{rd}, subject were to be treated at the same dose to confirm intolerance. If no safety concerns were identified and no Grade 3 or 4 AEs occurred in these subjects, dose escalation was to continue. When appropriate concentrations of drug were achieved, 1-2 additional subjects were treated at the same dose to verify results. After an overnight (8-hour) fast, each subject received a single oral dose of 5-azacytidine. Serial blood samples for plasma PK analyses were drawn before, and at the following time points after, dosing: 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 8 hours, and 10 and 12 hours (if possible). A validated high-performance liquid chromatography/tandem mass spectrometric method (LC-MS/MS) was used to determine 5-azacytidine concentrations in plasma. PK parameters calculated from plasma concentrations included (but were not limited to) C_{max}, T_{max}, $t_{½}$, and $AUC_{(0-\infty)}$.

[00146] **Patients**

[00147] Inclusion criteria:

a. Male or female subjects with MDS, AML, or malignant solid tumors, ≥18 years of age, with Eastern Cooperative Oncology Group (ECOG) performance status 0 – 2 were eligible.
b. For subjects with AML or malignant solid tumors, eligibility was limited to those for whom standard curative or palliative measures did not exist or were no longer effective.
c. Patients must have had normal renal, hepatic, and gastrointestinal function.

[00148] Exclusion criteria:

a. Pregnancy
b. History of severe cardiac or pulmonary disease

c. Advanced malignant hepatic tumor

d. Receipt of radiation therapy, chemotherapy, or investigational drugs within 30 days of planned testing.

[00149] **Patients** (Demographic and disease characteristics are shown in Table 4.)

[00150] 4 subjects were enrolled and received study drug

a. 1 subject received a 60 mg dose which was well tolerated. 5-azacytidine was quantifiable in plasma in 2 samples and the dose was escalated.

b. 1 subject received an 80 mg dose which was well tolerated. 5-azacytidine was detectable in 4 consecutive samples. Two additional subjects were then treated at the 80 mg level.

[00151] **Table 4**

Subject (pt. #)	Age	Sex	Date of Diagnosis	Tumor Type	ECOG Status	Oral Aza Dose
1 (101)	43	M	5/90	Metastatic thymic carcinoid, mets to lung and skin lesions	1 (restricted)	60 mg
2 (102)	67	M	12/00	Prostate cancer	0 (fully active)	80 mg
3 (201)	57	M	12/06	AML	2 (ambulatory, capable of self care)	80 mg
4 (202)	65	M	11/06	MDS secondary to successfully treated AML (CR achieved)	1 (restricted)	80 mg

[00152] PK Results

[00153] Concentration vs time profiles of individual subjects are shown in **Figure 5** (semi-logarithmic scale)

a. C_{max} for subjects who received an 80 mg dose were approximately 2-, 5-, and 6-fold higher than that of the subject dosed at 60 mg.

b. Bioavailability of the 80 mg oral dose relative to SC dosing was 6.3%, 24%, and 22%.

c. T_{max} for subjects who received an 80 mg dose occurred at 1.5, 2.0, and 1.0 h post-dose.

[00154] 5-azacytidine plasma concentrations for each subject, the mean concentration of the 80 mg dose group (n=3), and mean SC 5-azacytidine concentrations (historical data; n=6) are presented in Table 5.

[00155] **Table 5: 5-azacytidine Plasma Concentrations (ng/mL)**

Oral Azacitidine Concentrations (ng/mL)						SC Azacitidine Concentrations (ng/mL)	
	Subject #					Time (h)	75 mg/m² SC
Time (h)	101 (80 mg)	102 (80 mg)	201 (80 mg)	202 (80 mg)	Mean (80 mg)		
0	0	0	0	0	0	0	0
0.5	BLQ	BLQ	BLQ	4.46	1.49	0.5	750
1.0	3.72	9.25	31.5	99.1	46.6	1	354.2
1.5	4.56	34.3	58.5	66.7	53.2	2	124.5
2.0	3.75	11	75.1	24.5	36.9	4	17.9
2.5	12.4	3.61	36.4	12.5	17.5	8	BLQ
3.0	15.8	1.4	14.1	3.41	6.30		
3.5	4.27	BLQ	6.1	1.61	2.57		
4.0	1.55	BLQ	2.43	BLQ	BLQ		
4.5	BLQ	BLQ	1.19	BLQ	BLQ		
5.0	BLQ	BLQ	BLQ	BLQ	BLQ		
BLQ=below limit of quantitation							

[00156] Concentration vs time profiles for the 60 mg dose and the mean of the three 80 mg doses are shown in **Figure 6** (semi-logarithmic scale).

[00157] For the mean 80 mg dose:

a. Concentrations were below the level of quantitation at 4.0 h

b. T_{max} was 1.5 h

c. C_{max} increased 3.5-fold versus the 60 mg dose

d. Bioavailability relative to the historical SC 5-azacytidine group was 18%.

[00158] A summary of results of PK measurements is presented in Table 6.

[00159] **Table 6**:

Subject #	Dose	$AUC_{(0-\infty)}$ (ng*h/mL)	C_{max} (ng/mL)	$t_{1/2}$ (h)	T_{max} (h)	F (%)*
101	60 mg	22.6	15.8	0.299	3.0	6.7
102	80 mg	28.6	34.3	0.336	1.5	6.3
201	80 mg	111	75.1	0.416	2.0	24
202	80 mg	103	99.1	0.366	1.0	22
Mean (n=3)	80 mg	81.5	53.2	0.361	1.5	18
*Percent bioavailability compared with historical SC azacitidine data (dose = 135 mg; AUC = 777 ng*h/mL)						

[00160] Study endpoint was reached following evaluation of 4 subjects.

[00161] Safety

[00162] All AEs possibly related to study drug were Grade 2. No study-drug-related SAEs were reported.

[00163] The outcome from the clinical trial was shown to be that patients dosed with the the referenced oral 5-azacytidine formulation showed measurable levels of 5-azacytidine in plasma samples. The amount of 5-azacytidine measured in the plasma was proportional to the administered dose, and the apparent oral bioavailability is acceptable for therapeutic treatment. The conclusions drawn from such results are that the current formulation delivered 5-azacytidine to the colonic region, and that site was shown to be capable of efficiently absorbing 5-azacytidine without the degradation associated with cytidine deaminase.

Example 6

[00164] Solid Oral Dosage Form

[00165] Solid oral dosage forms of 5-azacytidine were prepared using standard pharmaceutical excipients and techniques. TPGS was first adsorbed onto either microcrystalline cellulose or calcium silicate in an independent step. Dry ingredients were then dry blended and tablets prepared by direct compression. Tablets were then film coated with Klucel EF from ethanol followed by film coating with a mixture of Eudragit RS PO, triethyl citrate, pectin and chitosan from an ethanol – acetone mixture. Eudragit RS PO is a water insoluble copolymer of methacrylic acid and aminoethylmethacrylic acid which has low permeability and pH independent swelling characteristics. Its inclusion in the film coat is to facilitate film formation rather than provide a pH dependent barrier to dissolution Pectin – chitosan complex was prepared via neutralization of an acidic 1:1 mixture of aqueous pectin and chitosan followed by collection and drying of the prepared solid.

[00166] Clinical Trial Material cores were composed of the following materials in these ratios:

Ingredient	**mg/tablet**	**% w/w**
5-azacytidine	20.0	20.0
Mannitol, USP	43.2	43.2
Microcrystalline Cellulose, NF	30.0	30.0
Crospovidone, NF	3.0	3.0

Magnesium Stearate, NF	1.8	1.8
Vitamin E TPGS, NF	2.0	2.0

[00167] Cores were then sub-coated to approximately 4% w/w with the following materials in ethanol:

Ingredient	**mg/tablet**	**% w/w**
Klucel EF, NF	4.0	6.0

[00168] Film coated cores were then be coated to approximately 9% w/w with the following mixture:

Ingredient	**mg/tablet**	**% w/w**
Eudragit RS PO, NF	5.0	55.6
Triethyl citrate, NF	1.0	11.2
Pectin, USP	1.5	16.6
Chitosan, Low MW	1.5	16.6

[00169] Excipient compatibility studies have demonstrated that 5-azacytidine is compatible with each excipient. Stability studies have demonstrated excellent stability of coated tablets under long term (25°C, 60% RH) and accelerated (40°, 70% RH) storage conditions. An additional formulation composition that has been manufactured is presented in Table 7.

[00170] **Table 7: Oral 5-azacytidine Tablet Composition**

Material	Trade Name	Purpose	Quality Standard
5-azacytidine	NA	Active	In-House
Mannitol	Parteck M200	Bulking Agent	USP
Microcrystalline Cellulose	Prosolv 90HD	Binding Agent	USP
Crospovidone	Polyplasone XL	Disintegrant	USP
Magnesium Stearate	NA	Lubricant	USP
Vitamin E TPGS	NA	Absorption Enhancement	NF
Hydroxypropyl Cellulose	Klucel EF	Sub-Coating	NF
Methacrylic acid copolymer	Eudragit RS PO	Film Coating	USP
Triethyl Citrate	Morflex	Plasticizer	USP
Pectin	CP Kelco Kelcogel	Colonic Selective Releasing Agent	USP
Chitosan, Low MW	NA	Colonic Selective Releasing Agent	In-House

Example 7

[00171] Solid Oral Dosage Form

[00172] Solid oral dosage forms of 5-azacytidine were prepared using standard pharmaceutical excipients and techniques. TPGS was first adsorbed onto either microcrystalline cellulose or calcium silicate in an independent step. Dry ingredients were then dry blended and tablets prepared by direct compression. Tablets were then film coated with Klucel EF from ethanol followed by film coating with a mixture of Eudragit RS PO, triethyl citrate, and amylose acetate from an ethanol – diethyl ether mixture. Eudragit RS PO is a water insoluble copolymer of methacrylic acid and aminoethylmethacrylic acid which has low permeability and pH independent swelling characteristics. Its inclusion in the film coat is to facilitate film formation rather than provide a pH dependent barrier to dissolution.

[00173] Clinical Trial Material cores were composed of the following materials in these ratios:

Ingredient	**mg/tablet**	**% w/w**
5-azacytidine	20.0	20.0
Mannitol, USP	43.2	43.2
Microcrystalline Cellulose, NF	30.0	30.0
Crospovidone, NF	3.0	3.0
Magnesium Stearate, NF	1.8	1.8
Vitamin E TPGS, NF	2.0	2.0

[00174] Cores were then sub-coated to approximately 4% w/w with the following materials in ethanol:

Ingredient	**mg/tablet**	**% w/w**
Klucel EF, NF	4.0	6.0

[00175] Film coated cores were then be coated to approximately 9% w/w with the following mixture:

Ingredient	**mg/tablet**	**% w/w**
Eudragit RS PO, NF	4.0	44.5
Triethyl citrate, NF	0.75	8.3
Amylose acetate	4.25	47.2

[00176] Excipient compatibility studies have demonstrated that 5-azacytidine is compatible with each excipient. Stability studies have demonstrated excellent stability of coated tablets under long term (25°C, 60% RH) and accelerated (40°, 70% RH) storage conditions. An additional formulation composition that has been manufactured is presented in Table 8.

[00177] **Table 8: Oral 5-azacytidine Tablet Composition**

Material	Trade Name	Purpose	Quality Standard
5-azacytidine	NA	Active	In-House
Mannitol	Parteck M200	Bulking Agent	USP
Microcrystalline Cellulose	Prosolv 90HD	Binding Agent	USP
Crospovidone	Polyplasone XL	Disintegrant	USP
Magnesium Stearate	NA	Lubricant	USP
Vitamin E TPGS	NA	Absorption Enhancement	NF
Hydroxypropyl Cellulose	Klucel EF	Sub-Coating	NF
Methacrylic acid copolymer	Eudragit RS PO	Film Coating	USP
Triethyl Citrate	Morflex	Plasticizer	USP
Amylose acetate	NA	Colonic Selective Releasing Agent	In-House

Example 8

[00178] Solid Oral Dosage Form

[00179] Solid oral dosage forms of 5-azacytidine were prepared using standard pharmaceutical excipients and techniques. TPGS was first adsorbed onto either microcrystalline cellulose or calcium silicate in an independent step. Dry ingredients were then dry blended and tablets prepared by direct compression. Tablets were then film coated with Klucel EF from ethanol followed by film coating with a polymer of 2-hydroxyethyl methacrylic acid cross linked with divinyl azobenzene (HEMA-DVAB polymer) and triethyl citrate from an ethanol – diethyl ether solvent mixture.

[00180] Clinical Trial Material cores were composed of the following materials in these ratios:

Ingredient	mg/tablet	% w/w
5-azacytidine	20.0	20.0
Mannitol, USP	43.2	43.2

Microcrystalline Cellulose, NF	30.0	30.0
Crospovidone, NF	3.0	3.0
Magnesium Stearate, NF	1.8	1.8
Vitamin E TPGS, NF	2.0	2.0

[00181] Cores were then sub-coated to approximately 4% w/w with the following materials in ethanol:

Ingredient	**mg/tablet**	**% w/w**
Klucel EF, NF	4.0	6.0

[00182] Film coated cores were then be coated to approximately 6% w/w with the following mixture:

Ingredient	**mg/tablet**	**% w/w**
HEMA-DVAB Polymer	5.0	83.3
Triethyl citrate, NF	1.0	16.7

[00183] Excipient compatibility studies have demonstrated that 5-azacytidine is compatible with each excipient. Stability studies have demonstrated excellent stability of coated tablets under long term (25°C, 60% RH) and accelerated (40°, 70% RH) storage conditions. An additional formulation composition that has been manufactured is presented in Table 9.

[00184] **Table 9: Oral 5-azacytidine Tablet Composition**

Material	Trade Name	Purpose	Quality Standard
5-azacytidine	NA	Active	In-House
Mannitol	Parteck M200	Bulking Agent	USP
Microcrystalline Cellulose	Prosolv 90HD	Binding Agent	USP
Crospovidone	Polyplasone XL	Disintegrant	USP
Magnesium Stearate	NA	Lubricant	USP
Vitamin E TPGS	NA	Absorption Enhancement	NF
Hydroxypropyl Cellulose	Klucel EF	Sub-Coating	NF
HEMA-DVAB polymer	NA	Colonic Selective Releasing Agent	In-House
Triethyl Citrate	Morflex	Plasticizer	USP

Example 9

[00185] Solid Oral Dosage Form

[00186] Solid oral dosage forms of 5-azacytidine were prepared using standard pharmaceutical excipients and techniques. TPGS was first adsorbed onto either microcrystalline cellulose or calcium silicate in an independent step. Dry ingredients were then dry blended for subsequent encapsulation into water impermeable, crosslinked gelatin capsules. The open capsule end was then sealed with Eudragit RL PO, a water insoluble, pH independent, swelling polymethacrylate polymer and triethyl citrate from ethanol. The amount of Eudragit RL PO polymer used to seal the capsule end was sufficient to require 3 hours or exposure to water prior to release of the capsule contents.

[00187] Clinical Trial Material capsules were composed of the following materials in these ratios:

Ingredient	**mg/capsule**	**% w/w**
5-azacytidine	20.0	20.0
Mannitol, USP	33.2	33.2
Microcrystalline Cellulose, NF	40.0	40.0
Crospovidone, NF	3.0	3.0
Magnesium Stearate, NF	1.8	1.8
Vitamin E TPGS, NF	2.0	2.0

[00188] Filled capsules were then sealed with approximately 11.5% w/w the following mixture of Eudragit RL PO/triethyl citrate:

Ingredient	**mg/capsule**	**% w/w**
Eudragit RL PO, NF	10.0	87.0
Triethyl citrate, NF	1.5	13.0

[00189] Excipient compatibility studies have demonstrated that 5-azacytidine is compatible with each excipient. Stability studies have demonstrated excellent stability of coated tablets under long term (25°C, 60% RH) and accelerated (40°, 70% RH) storage conditions. An additional formulation composition that has been manufactured is presented in Table 10.

[00190] Table 10: Oral 5-azacytidine Tablet Composition

Material	Trade Name	Purpose	Quality Standard
5-azacytidine	NA	Active	In-House
Mannitol	Parteck M200	Bulking Agent	USP
Microcrystalline Cellulose	Prosolv 90HD	Binding Agent	USP
Crospovidone	Polyplasone XL	Disintegrant	USP
Magnesium Stearate	NA	Lubricant	USP
Vitamin E TPGS	NA	Absorption Enhancement	NF
Hydroxypropyl Cellulose	Klucel EF	Sub-Coating	NF
Methacrylic acid copolymer[1]	Eudragit RL PO	Film Coating	USP
Triethyl Citrate	Morflex	Plasticizer	USP

Example 10

[00191] A clinical study using each of the oral formulations described in Examples 6-9 is performed to assess the safety and bioavailability of single oral doses of 5-azacytidine in patients with myelodysplastic syndromes, acute myelogenous leukemia, or solid tumors. The study is a multicenter, open-label, single treatment study. One patient receives an oral dose of the study drug starting at 60 mg. Subsequent patients are treated with escalating doses, in 20 mg increments, up to 200 mg. The study assesses the safety and tolerability of escalating doses, provides pilot information on the oral bioavailability of the study drug, and provides information on the single dose pharmacokinetics of the study drug after oral administration.

[00192] The outcome from the clinical trial is shown to be that patients dosed with the current oral 5-azacytidine formulation show measurable levels of 5-azacytidine in plasma samples. The amount of 5-azacytidine measured in the plasma is proportional to the administered dose, and the apparent oral bioavailability is acceptable for therapeutic treatment. The conclusions drawn from such results are that the current formulation is delivering 5-azacytidine to the colonic region, and that site is capable of efficiently absorbing 5-azacytidine without the degradation associated with cytidine deaminase. Ultimately, such observations lead to broader clinical applications of 5-azacytidine.

[00193] While the invention has 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 without departing

from the spirit and scope of the invention and that the various embodiments disclosed herein are not intended to act as limitations on the scope of the claims.

CLAIMS

What is claimed is:

1. A controlled release pharmaceutical composition for oral administration of a cytidine analog comprising a) a therapeutically effective amount of a cytidine analog and b) a drug release controlling component capable of providing release of the cytidine analog primarily in the large intestine, wherein after ingestion by a patient the cytidine analog is released primarily in the large intestine.

2. The pharmaceutical composition of claim 1, wherein at least about 70% of the cytidine analog is released in the large intestine.

3. The pharmaceutical composition of claim 1, wherein the drug release controlling component is selected from the group consisting of an enteric component, a time delay component, a bacterially degradable component, and mixtures thereof.

4. The pharmaceutical composition of claim 3, wherein the drug release controlling component is an enteric coating, and wherein the enteric coating does not substantially dissolve in aqueous solution at a pH of above about pH 6.4 for at least about two hours.

5. The pharmaceutical composition of claim 3, wherein the enteric coating material comprises an agent selected from the group consisting of any grade of hydroxypropylmethylcellulose phthalate, polyvinyl acetate phthalate (PVAP), hydroxypropylmethylcellulose acetate succinate (HPMCAS), alginate, carbomer, carboxymethyl cellulose, methacrylic acid copolymer, shellac, cellulose acetate phthalate (CAP), starch glycolate, polacrylin, methyl cellulose acetate phthalate, hydroxylmethylcellulose phthalate, hydroxymethylmethylcellulose acetate succinate, hydroxypropylcellulose acetate phthalate, cellulose acetate terephthalate, cellulose acetate isophthalate, cellulose acetate trimellitate, and mixtures thereof.

6. The pharmaceutical composition of claim 5, wherein the methacrylic acid copolymer is selected from the group consisting of a cationic copolymer of dimethyl aminoethyl methacrylate and neutral methacrylic esters, trimethylaminoethylmethacrylate and neutral methacrylic esters, and anionic polymers of methacrylic acid and methacrylates with carboxyl functional groups.

7. The pharmaceutical composition of claim 4, comprising

(a) a drug core and seal coat, wherein the drug core comprises 5-azacytidine, in an amount of at least about 20% w/w of the drug core and seal coat, further comprising at least one of the following excipients: diluent, binding agent, lubricant, disintegrant, and stabilizer and further comprising a seal coat, in an amount sufficient to form a sealed drug core; and

(b) an enteric coat, in an additional amount of between about 2% and 20% w/w relative to the drug core and seal coat, wherein the enteric coat comprises an anionic polymer of methacrylic acid and methacrylates with carboxyl functional groups with a threshold pH of about 6.8(EUDRAGIT S100), in an amount of between about 60% and about 95 % w/w of the enteric coat, and optionally further comprising a plasticizer, in an amount of between about 5% and about 40% w/w of the enteric coat.

8. The pharmaceutical composition of claim 7, wherein the excipients comprise at least one of the following: (a) a diluent, wherein the diluent comprises mannitol, in an amount of about 43% w/w of the drug core and seal coat; (b) a binding agent, wherein the binding agent comprises microcrystalline cellulose, in an amount of about 30% w/w of the drug core and seal coat; (c) a disintegrant, wherein the disintegrant comprises crospovidone, in an amount of about 3% w/w of the drug core and seal coat; (d) a lubricant, wherein the lubricant comprises magnesium stearate, in an amount of about 1.8% w/w of the drug core and seal coat, (e) a stabilizer, wherein the stabilizer comprises Vitamin E TPGS, in an amount of about 2% w/w of the drug core and seal coat; wherein the seal coat comprises hydroxypropyl cellulose, in an amount of about 6% w/w of the drug core and seal coat; and wherein the enteric coat comprises an additional amount of about 7% w/w relative to the drug core and seal coat, and the anionic polymer of methacrylic acid and methacrylates with carboxyl functional groups with a threshold pH of about 6.8, in an amount of about 86% w/w of the enteric coat and wherein the enteric coat

further comprises a plasticizer comprising triethyl citrate, in an amount of about 14% w/w of the enteric coat.

9. The controlled release pharmaceutical composition of claim 3, wherein the drug release controlling component comprises a time delay component, and wherein the time delay component does not allow substantial release of the cytidine analog for at least about three hours after oral ingestion by a patient.

10. The controlled release pharmaceutical composition of claim 9, wherein the time delay component is a matrix or coating and is selected from the group consisting a poorly soluble polymer selected from the group consisting of polyvinyl chloride, polyethylene, vinyl polymers and copolymers selected from the group consisting of polyvinyl pyrrolidone, polyvinyl acetate, polyvinylacetate phthalate, vinylacetate crotonic acid copolymer, and ethylene-vinyl acetate copolymer; hydroxypropyl methyl cellulose, shellac, ammoniated shellac, shellac-acetyl alcohol, shellac n-butyl stearate, and copolymers of acrylic and methacrylic acid esters with a low content in quaternary ammonium groups with an average molecular weight of about 150,000 D (EUDRAGIT RS PO).

11. The pharmaceutical composition of claim 9, comprising

(a) a drug core and seal coat, wherein the drug core comprises 5-azacytidine, in an amount of at least about 20% w/w of the drug core and seal coat, further comprising at least one of the following excipients: diluent, binding agent, lubricant, disintegrant, stabilizer; and further comprising a seal coat, in an amount sufficient to form a sealed drug core; and

(b) a time delay coat, in an additional amount of between about 2% and about 20% w/w relative to the drug core and seal coat, wherein the time delay coat comprises copolymers of acrylic and methacrylic acid esters with a low content in quaternary ammonium groups with an average molecular weight of about 150,000 D (EUDRAGIT RS PO), in an amount of between about 60 % and about 95% w/w of the time delay coat, and optionally further comprising a plasticizer, in an amount of between about 5% and 40% w/w of the time delay coat.

12. The pharmaceutical composition of claim 11, wherein the excipients comprise at least one of the following: (a) a diluent, wherein the diluent comprises mannitol, in an amount of about 33% w/w of the drug core and seal coat; (b) a binding agent, wherein the binding agent comprises microcrystalline cellulose, in an amount of about 40% w/w of the drug core and seal coat; (c) a disintegrant, wherein the disintegrant comprises crospovidone, in an amount of about 3% w/w of the drug core and seal coat; (d) a lubricant, wherein the lubricant comprises magnesium stearate, in an amount of about 1.8% w/w of the drug core and seal coat, (e) a stabilizer, wherein the stabilizer comprises Vitamin E TPGS, in an amount of about 2% w/w of the drug core and seal coat; wherein the seal coat comprises hydroxypropyl cellulose, in an amount of about 5% w/w of the drug core and seal coat; and wherein the time coat comprises an additional amount of about 11.5% w/w relative to the drug core and seal coat, and wherein the copolymers of acrylic and methacrylic acid esters with a low content in quaternary ammonium groups with an average molecular weight of about 150,000 D (EUDRAGIT RS PO) are in an amount of about 87% w/w of the time delay coat and wherein the coat further comprises a plasticizer, wherein the plasticizer is triethyl citrate, in an amount of about 13% w/w of the time delay coat.

13. The controlled release pharmaceutical composition of claim 3, wherein the drug release controlling component comprises a bacterially degradable component, wherein patients lack the digestive enzymes required to degrade the component.

14. The controlled release pharmaceutical component of claim 13, wherein the bacterially degradable component is selected from the group consisting of a polymer of 2-hydroxyethyl methacrylic acid cross linked with divinyl azobenzene (HEMA-DVAB polymer), chitosan, amylose, cellobiose, lactulose, raffinose and stachyose, and polymers thereof.

15. The pharmaceutical composition of claim 9, comprising

(a) a drug core and seal coat, wherein the drug core comprises 5-azacytidine, in an amount of at least about 20% w/w of the drug core and seal coat, and further comprising at least one of the following excipients: diluent, binding agent, lubricant, disintegrant, stabilizer, and further comprising a seal coat, in an amount sufficient to form a sealed drug core; and

(b) a bacterially degradable coat, in an additional amount of between about 2% and 20% w/w relative to the drug core and seal coat, wherein the bacterially degradable coat comprises one of the following formulations:

(i) copolymers of acrylic and methacrylic acid esters with a low content in quaternary ammonium groups with an average molecular weight of about 150,000 D (EUDRAGIT RS PO), in an amount of between about 30% and about 70% w/w of the bacterially degradable coat; optionally further comprising a plasticizer, in an amount of between about 5% and 40% w/w of the bacterially degradable coat; pectin, in an amount of between about 10% and about 30% w/w of the bacterially degradable coat, and chitosan, in an amount of between about 10% and about 30% w/w of the bacterially degradable coat; and

(ii) copolymers of acrylic and methacrylic acid esters with a low content in quaternary ammonium groups with an average molecular weight of about 150,000 D (EUDRAGIT RS PO), in an amount of between about 30% and about 70% w/w of the bacterially degradable coat; optionally further comprising a plasticizer, in an amount of between about 5% and 40% w/w of the bacterially degradable coat; and amylose, in an amount of between about 30% and about 60% w/w of the bacterially degradable coat.

16. The pharmaceutical composition of claim 15, wherein the excipients comprise at least one of the following (a) diluent, wherein the diluent comprises mannitol, in an amount of about 43% w/w of the drug core and seal coat; (b) a binding agent, wherein the binding agent comprises microcrystalline cellulose, in an amount of about 30% w/w of the drug core and seal coat; (c) disintegrant, wherein the disintegrant comprises crospovidone, in an amount of about 3% w/w of the drug core and seal coat; (d) lubricant, wherein the lubricant comprises magnesium stearate, in an amount of about 1.8% w/w of the drug core and seal coat, (e) stabilizer, wherein the stabilizer comprises Vitamin E TPGS, in an amount of about 2% w/w of the drug core and seal coat; further comprising a seal coat, wherein the seal coat comprises hydroxypropyl cellulose, in an amount of about 4% w/w of the drug core and seal coat; and wherein the bacterially degradable coat in (i) comprises an additional amount of about 9% w/w relative to the drug core and seal coat of a mixture of copolymers of acrylic and methacrylic acid esters with a low content in quaternary ammonium groups with an average molecular weight of about 150,000 D (EUDRAGIT RS PO) in an amount of about 56% w/w of the bacterially degradable coat, a

plasticizer, wherein the plasticizer is triethyl citrate in an amount of about 11% w/w of the bacterially degradable coat, pectin, in an amount of about 17% w/w of the bacterially degradable coat; and chitosan, in an amount of about 17% w/w of the bacterially degradable coat; and wherein the bacterially degradable coat in (ii) comprises an additional amount of about 9% w/w relative to the drug core and seal coat of a mixture of copolymers of acrylic and methacrylic acid esters with a low content in quaternary ammonium groups with an average molecular weight of about 150,000 D (EUDRAGIT RS PO) in an amount of about 45% w/w of the bacterially degradable coat, a plasticizer, wherein the plasticizer is triethyl citrate in an amount of about 8% w/w of the bacterially degradable coat, and amylose, in an amount of about 47% w/w of the bacterially degradable coat.

17. The pharmaceutical composition of claim 9, comprising

(a) a drug core and seal coat, wherein the drug core comprises 5-azacytidine, in an amount of least about 20% w/w of the drug core and seal coat, further comprising at least one of the following excipients: diluent, binding agent, lubricant, disintegrant, stabilizer and further comprising a a seal coat, in an amount sufficient to form a sealed drug core; and

(c) a bacterially degradable coat, in an additional amount of between about 2% and about 20% w/w relative to the drug core and seal coat, wherein the bacterially degradable coat comprises a polymer of 2-hydroxyethyl methacrylic acid cross linked with divinyl azobenzene (HEMA-DVAB polymer), in an amount of between about 60% and about 95% w/w of the bacterially degradable coat, and optionally further comprising a plasticizer, in an amount of between about 5% and about 40% w/w of the bacterially degradable coat.

18. The pharmaceutical composition of claim 17, wherein the excipients comprise at least one of the following: (a) a diluent, wherein the diluent comprises mannitol, in an amount of about 43% w/w of the drug core and seal coat, (b) a binding agent, wherein the binding agent comprises microcrystalline cellulose, in an amount of about 30% w/w of the drug core and seal coat; (c) disintegrant, wherein the disintegrant comprises crospovidone, in an amount of about 3% w/w of the drug core and seal coat, (d) lubricant, wherein the lubricant comprises magnesium stearate, in an amount of about 1.8% w/w of the drug core and seal coat, (e) stabilizer, wherein the stabilizer comprises Vitamin E TPGS, in an amount of about 2% w/w of the drug core and

seal coat; wherein the seal coat comprises hydroxypropyl cellulose, in an amount of about 4% w/w of the drug core and seal coat; and wherein the bacterially degradable coat is in an additional amount about 6% w/w relative to the drug core and seal coat, and the polymer of 2-hydroxyethyl methacrylic acid cross linked with divinyl azobenzene (HEMA-DVAB polymer) in an amount of about 83% w/w of the bacterially degradable coat, and wherein the bacterially degradable coat further comprises a plasticizer, wherein the plasticizer is triethyl citrate in an amount of about 17% w/w of the bacterially degradable coat.

19. The pharmaceutical composition of claim 1, wherein the cytidine analog is selected from the group consisting of 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(1H) pyrimidine riboside (also called zebularine), 2'-cyclocytidine, arabinofuanosyl-5-azacytidine, dihydro-5-azacytidine, N^4-octadecyl-cytarabine, and elaidic acid cytarabine.

20. The pharmaceutical composition of claim 19, wherein the cytidine analog is 5-azacytidine.

21. A method for treating a patient having a disease associated with abnormal cell proliferation, comprising: orally administering to the patient a pharmaceutical composition in accordance with claim 1.

22. The method of claim 21, wherein the disease associated with abnormal cell proliferation is a myelodysplastic syndrome.

23. A method for delivering a cytidine analog comprising administering to a patient in need thereof an oral formulation of a cytidine analog, wherein the oral formulation of the cytidine analog comprises a) a therapeutically effective amount of a cytidine analog and b) a drug release controlling component capable of providing release of the cytidine analog primarily in the large intestine, wherein after ingestion by a patient the cytidine analog is released primarily in the large intestine.

24. The method of claim 23, wherein the drug release controlling component is selected from the group consisting of an enteric component, a time delay component, a bacterially degradable component, and mixtures thereof.

25. The method of claim 23, wherein the drug release controlling component is an enteric coating, and wherein the enteric coating does not substantially dissolve in aqueous solution at a pH of above about pH 6.4 for at least about two hours.

26. The method of claim 23, wherein the drug release controlling component comprises a time delay component, and wherein the time delay component does not allow substantial release of the cytidine analog for at least about three hours after oral ingestion by a patient.

27. The method of claim 23, wherein the drug release controlling component comprises a bacterially degradable component, wherein patients lack the digestive enzymes required to degrade the component.

28. The method of claim 23, wherein the cytidine analog is 5-azacytidine.

29. The method of claim 23, wherein the patient has a myelodysplastic syndrome.

30. A method of formulating a cytidine analog for oral delivery, comprising coating a therapeutically effective amount of a cytidine analog with a drug release controlling component capable of providing release of the cytidine analog primarily in the large intestine.

31. The method of claim 30, wherein the drug release controlling component is selected from the group consisting of an enteric component, a time delay component, a bacterially degradable component, and mixtures thereof.

32. The method of claim 30, wherein the drug release controlling component is an enteric coating, and wherein the enteric coating does not substantially dissolve in aqueous solution at a pH of above about pH 6.4 for at least about two hours.

33. The method of claim 30, wherein the drug release controlling component comprises a time delay component, and wherein the time delay component does not allow substantial release of the cytidine analog for at least about three hours after oral ingestion by a patient.

34. The method of claim 30, wherein the drug release controlling component comprises a bacterially degradable component, wherein patients lack the digestive enzymes required to degrade the component.

35. The method of claim 30, wherein the cytidine analog is 5-azacytidine.

36. A method of increasing the bioavailability of a cytidine analog upon administration to a patient, comprising: (I) providing a controlled release pharmaceutical composition to a patient, comprising a) a therapeutically effective amount of a cytidine analog and b) a drug release controlling component capable of providing release of the cytidine analog primarily in the large intestine, wherein after ingestion by a patient the cytidine analog is released primarily in the large intestine; and (II) ingesting of said composition by the patient, whereby said composition contacts the biological fluids of the patient's body and increases the bioavailability of the cytidine analog.

37. The method of claim 36, wherein the drug release controlling component is selected from the group consisting of an enteric component, a time delay component, a bacterially degradable component, and mixtures thereof.

38. The method of claim 36, wherein the drug release controlling component is an enteric coating, and wherein the enteric coating does not substantially dissolve in aqueous solution at a pH of above about pH 6.4 for at least about two hours.

39. The method of claim 36, wherein the drug release controlling component comprises a time delay component, and wherein the time delay component does not allow substantial release of the cytidine analog for at least about three hours after oral ingestion by a patient.

40. The method of claim 36, wherein the drug release controlling component comprises a bacterially degradable component, wherein patients lack the digestive enzymes required to degrade the component.

41. The method of claim 36, wherein the cytidine analog is 5-azacytidine.

42. The method of claim 36, wherein the patient has a myelodysplastic syndrome.

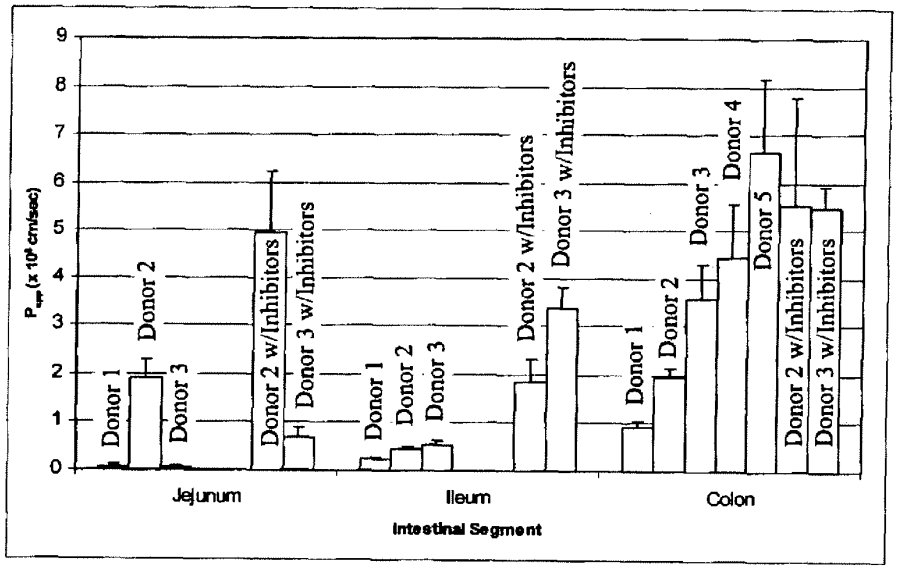


Fig. 1

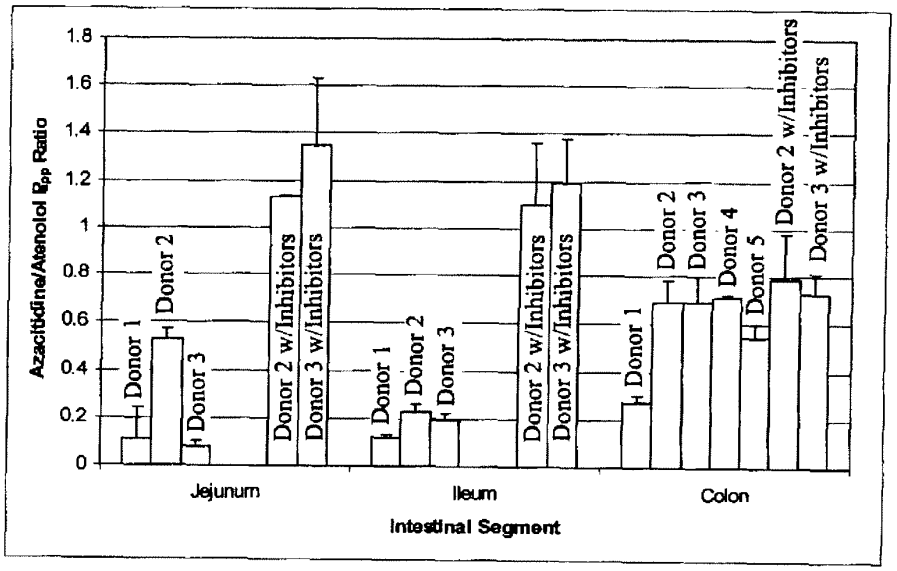


Fig. 2

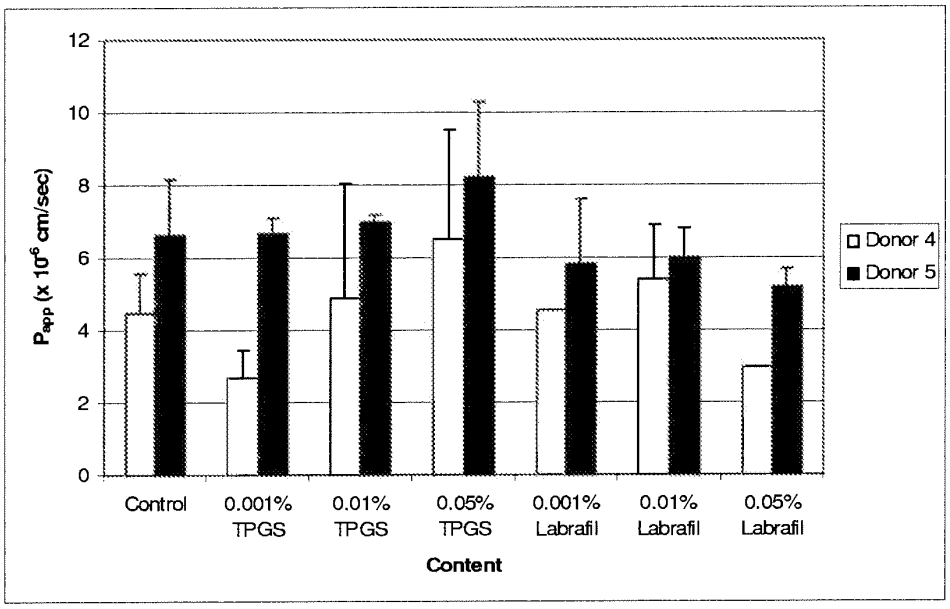


Fig. 3

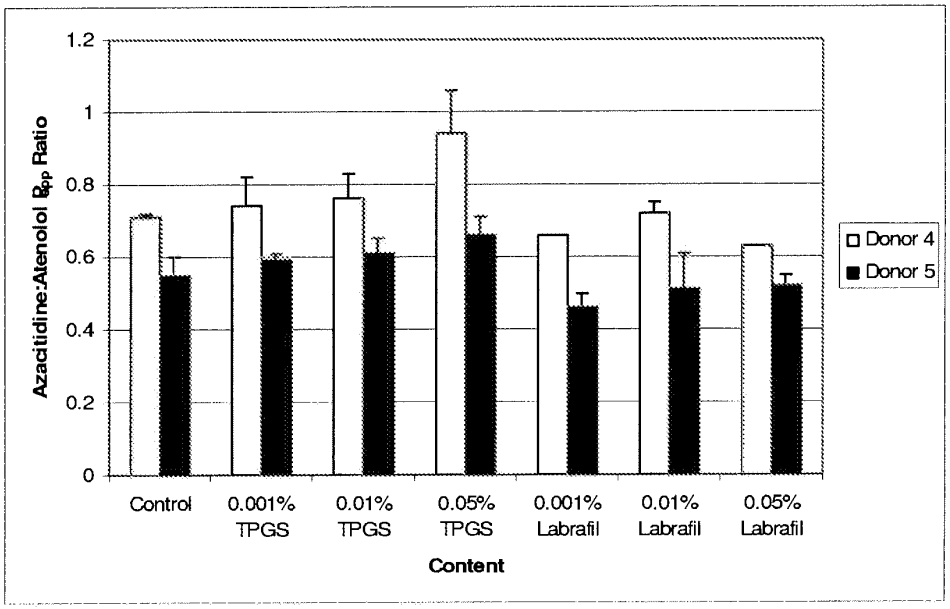


Fig. 4

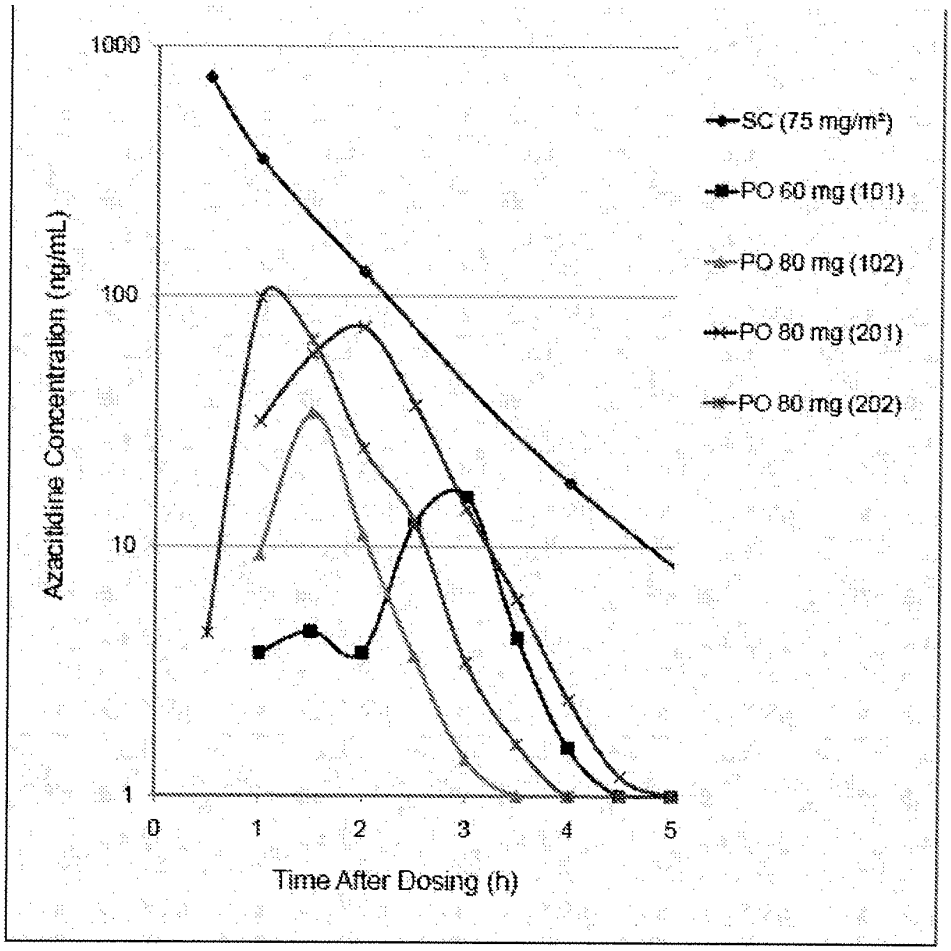


FIG. 5

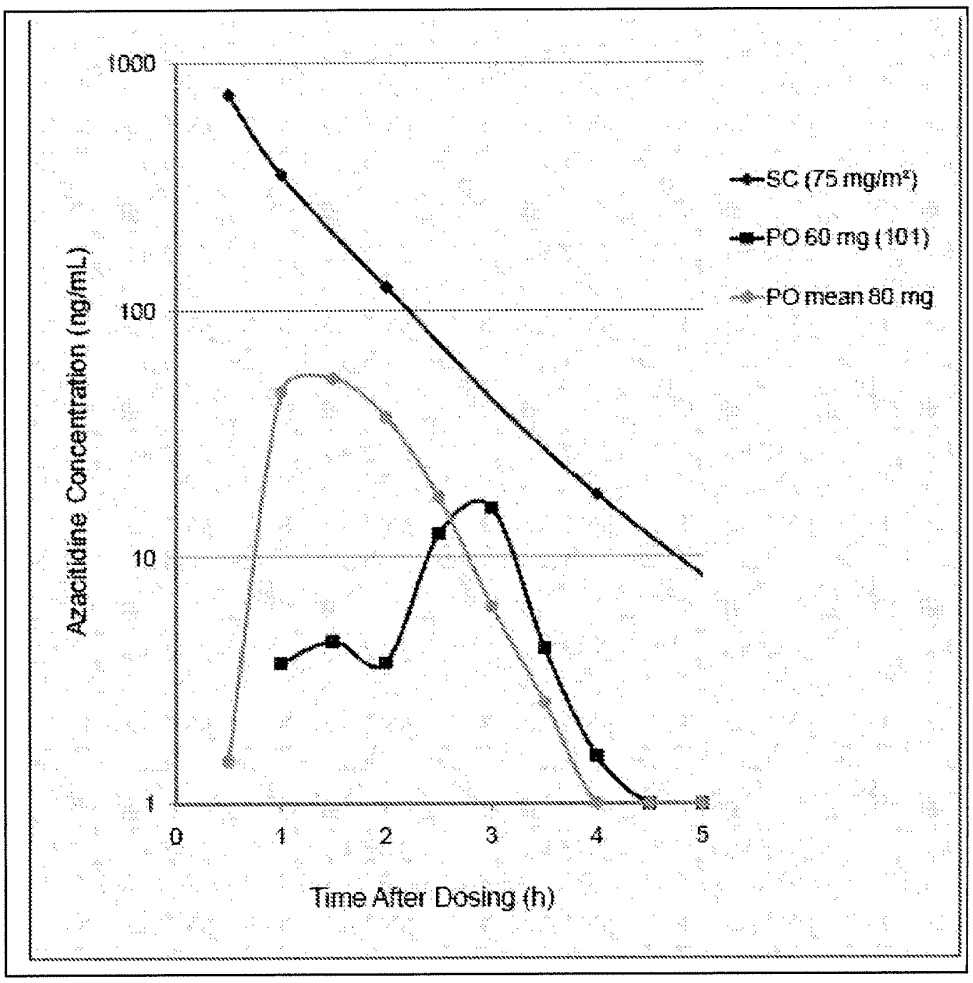


FIG. 6

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(54) Title: 2'-FLUOR0-2'-DEOXYTETRAHYDROURIDINES AS CYTIDINE DEAMINASE INHIBITORS

(57) Abstract: The present invention provides certain tetrahydrouridine derivative compounds, pharmaceutical compositions and kits comprising such compounds, and methods of making and using such compounds.

2'-FLUORO-2'-DEOXYTETRAHYDROURIDINES AS CYTIDINE DEAMINASE INHIBITORS

This application claims the benefit of U.S. Provisional Application No. 60/980,397, filed October 16, 2007, the entire contents of which is hereby incorporated by reference.

FIELD OF THE INVENTION

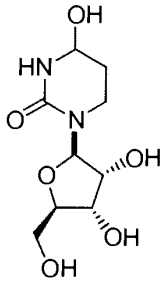
The present invention provides certain tetrahydrouridine derivative compounds which are inhibitors of the enzyme cytidine deaminase, pharmaceutical compositions and kits comprising such compounds, and methods of making and using such compounds.

BACKGROUND

The enzymes adenosine deaminase (ADA, EC 3.5.4.4) and cytidine deaminase (CDA, EC 3.5.4.5) function to deaminate natural aminopurine and aminopyrimidine nucleosides, respectively, in human and other organisms. They may also convert active nucleoside-based drugs into inactive metabolites. For example, the purine nucleoside drug arabinosyladenine (fludarabine, ara-A) is deaminated by ADA; the resulting compound, with the parent amino group replaced with hydroxyl, is inactive as an antitumor agent compared to the parent compound. Similarly, the antileukemia drug arabinosylcytosine (cytarabine, ara-C) is metabolically degraded by CDA into inactive arabinosyluracil.

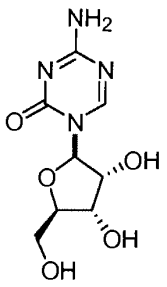
CDA is a component of the pyrimidine salvage pathway. It converts cytidine and deoxycytidine to uridine and deoxyuridine, respectively, by hydrolytic deamination (*Arch. Biochem. Biophys.* **1991**, *290*, 285-292; *Methods Enzymol.* **1978**, *51*, 401-407; *Biochem. J.* **1967**, *104*, 7P). It also deaminates a number of synthetic cytosine analogs which are clinically useful drugs, such as ara-C mentioned above (*Cancer Chemother. Pharmacol.* **1998**, *42*, 373-378; *Cancer Res.* **1989**, *49*, 3015-3019; *Antiviral Chem. Chemother.* **1990**, *1*, 255-262). Conversion of the cytosine compounds to the uridine derivatives usually confers loss of therapeutic activity or addition of side-effects. It has also been shown that cancers that acquire resistance to cytosine analog drugs often overexpress CDA (*Leuk. Res.* **1990**, *14*, 751-754). Leukemic cells expressing a high level of CDA can manifest resistance to cytosine antimetabolites and thereby limit the antineoplastic activity of such therapeutics (*Biochem. Pharmacol.* 1993, 45, 1857-1861). Inhibitors of CDA could therefore be useful adjuvants in combination chemotherapy.

Tetrahydrouridine (THU) has been known as an inhibitor of cytidine deaminase for a number of years.

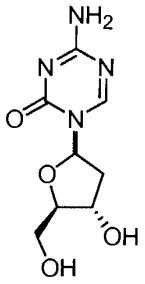


Tetrahydrouridine (THU)

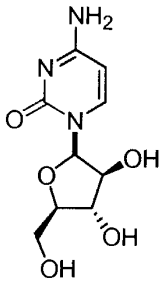
Various reports have suggested that co-administration with THU increases the efficacy and oral activity of cytidine-based drugs. For example, THU has been shown to enhance the oral activity of anti-leukemic agent 5-azacytidine in L1210 leukemic mice (*Cancer Chemotherapy Reports* **1975**, *59*, 459-465). The combination of THU plus 5-azacytidine has also been studied in a baboon sickle cell anemia model (*Am. J. Hematol.* **1985**, *18*, 283-288), and in human patients with sickle cell anemia in combination with orally administered 5-azacytidine (*Blood* **1985**, *66*, 527-532).



5-azacytidine



5-aza-2'-deoxycytidine (decitabine)



ara-C

THU has also been shown to enhance the oral efficacy of ara-C in L1210 leukemic mice (*Cancer Research* **1970**, *30*, 2166; *Cancer Invest* **1987**, 5, (4), 293-9), and in tumor-bearing mice (*Cancer Treat. Rep.* **1977**, *61*, 1355-1364). The combination of intravenously-administered ara-C with intravenously-administered THU has been investigated in several clinical studies in humans (*Cancer Treat. Rep.* **1977**, *61*, 1347-1353; *Cancer Treat. Rep.* **1979**, *63*, 1245-1249; *Cancer Res.* **1988**, *48*, 1337-1342). In particular, combination studies in

patients with acute myeloid leukemia (AML) and chronic myeloid leukemia (CML) have been performed (*Leukemia* **1991**, *5*, 991-998; *Cancer Chemother. Pharmacol.* **1993**, *31*, 481-484).

5-Aza-2'-deoxycytidine (decitabine) is an antineoplastic agent for the treatment of myelodysplastic syndrome (MDS), with potential utility for the treatment of AML and CML as well. Like the other cytidine-based drugs, its oral bioavailability and efficacy are limited by deactivation by CDA. THU has been shown to improve the potency of decitabine in a sickle cell disease model in baboons (*Am. J. Hematol.* **1985**, *18*, 283-288). In addition, another known CDA inhibitor, zebularine, has been shown to enhance the efficacy of decitabine in mice with L1210 leukemia (*Anticancer Drugs* **2005**, *16*, 301-308).

Gemcitabine, another cytidine-based antineoplastic drug, has also been studied in conjunction with CDA inhibitors (*Biochem. Pharmacol.* **1993**, *45*, 1857-1861). Co-administration with THU has been shown to alter the pharmacokinetics and bioavailability of gemcitabine in mice (*Abstr. 1556*, 2007 AACR Annual Meeting, April 14-18, 2007, Los Angeles, CA; *Clin. Cancer Res.* **2008**, *14*, 3529-3535).

5-Fluoro-2'-deoxycytidine (fluorocytidine, FdCyd) is another cytidine-based anticancer drug which is an inhibitor of DNA methyltransferase. The modulation of its metabolism and pharmacokinetics by THU in mice has been studied (*Clin Cancer Res.*, **2006**, *12*, 7483-7491; *Cancer Chemother. Pharm.* **2008**, *62*, 363-368).

The results of the aforementioned studies suggest that there is therapeutic utility in the administration of CDA inhibitors together with cytidine-based drugs such as ara-C, decitabine, 5-azacytidine and others. However, early CDA inhibitors such as THU suffer from drawbacks that include acid instability (*J. Med. Chem.* **1986**, *29*, 2351) and poor bioavailability (*J. Clin. Pharmacol.* **1978**, *18*, 259).

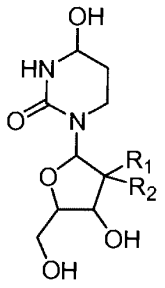
There is therefore an ongoing need for new, potent and therapeutically useful inhibitors of CDA.

BRIEF SUMMARY OF THE INVENTION

The present invention provides certain tetrahydrouridine derivative compounds, pharmaceutical compositions and kits comprising such compounds, and methods of making and using such compounds. The compounds, compositions, kits and methods of the invention may provide certain benefits. For example, the compounds and compositions of the invention may inhibit CDA enzyme activity and/or enhance the half-life, bioavailability and/or efficacy of drugs that are substrates for CDA. Additionally, the compounds, compositions, kits and

methods of the invention may exhibit improved aqueous solubility, chemical stability, drug absorption levels, toxicity levels, shelf-life, reproducibility in manufacturing and formulation, and therapeutic efficacy.

In one embodiment, the invention provides a compound of Formula I:



I

or a pharmaceutically acceptable salt of the compound, wherein:

R_1 and R_2 are independently selected from the group consisting of hydrogen, halo, cyano, nitro, sulfhydryl, hydroxyl, formyl, carboxyl, COO(C_1 to C_6 straight or branched chain alkyl), COO(C_1 to C_6 straight or branched chain alkenyl), COO(C_1 to C_6 straight or branched chain alkynyl), CO(C_1 to C_6 straight or branched chain alkyl), CO(C_1 to C_6 straight or branched chain alkenyl), CO(C_1 to C_6 straight or branched chain alkynyl), C_1 to C_6 straight or branched chain alkyl, C_1 to C_6 straight or branched chain alkenyl, C_1 to C_6 straight or branched chain alkynyl, C_1 to C_6 straight or branched chain alkoxy, and C_1 to C_6 straight or branched chain alkenoxy; wherein each occurrence of C_1 to C_6 straight or branched chain alkyl, C_1 to C_6 straight or branched chain alkenyl, C_1 to C_6 straight or branched chain alkynyl, C_1 to C_6 straight or branched chain alkoxy, or C_1 to C_6 straight or branched chain alkenoxy may be independently unsubstituted or substituted with one to four substituents independently selected from the group consisting of halo, hydroxyl, cyano, nitro, formyl, carboxyl, and sulfhydryl;

and provided that when one of R_1 and R_2 is -H, then the other is not -H, -OH or $-CH_2OH$.

In some embodiments, R_1 and R_2 are independently selected from the group consisting of hydrogen, halo, hydroxyl, cyano, nitro, sulfhydryl, C_1 to C_6 straight or branched chain alkyl, C_1 to C_6 straight or branched chain alkenyl, C_1 to C_6 straight or branched chain alkynyl, C_1 to C_6 straight or branched chain alkoxy, and C_1 to C_6 straight or branched chain alkenoxy; wherein wherein each occurrence of C_1 to C_6 straight or branched chain alkyl, C_1 to C_6 straight or branched chain alkenyl, C_1 to C_6 straight or branched chain alkynyl, C_1 to C_6

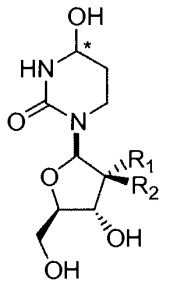
straight or branched chain alkoxy, or C_1 to C_6 straight or branched chain alkenoxy may be independently unsubstituted or substituted with one ore more halos;

and provided that when one of R_1 and R_2 is -H, then the other is not -H, or -OH.

In some embodiments, R_1 and R_2 are independently selected from the group consisting of hydrogen, halo, C_1 to C_6 alkyl, C_1 to C_6 alkenyl, C_1 to C_6 alkoxy, and C_1 to C_6 alkenoxy; wherein each occurrence of C_1 to C_6 straight or branched chain alkyl, C_1 to C_6 straight or branched chain alkenyl, C_1 to C_6 straight or branched chain alkoxy and C_1 to C_6 straight or branched chain alkenoxy may be independently unsubstituted or substituted with one to three halos;

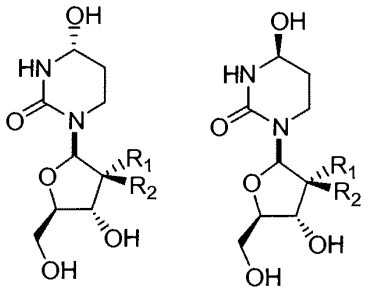
and provided that when one of R_1 and R_2 is -H, then the other is not -H, or -OH.

In some embodiments, the compound of Formula is:



wherein the carbon marked by an asterisk may have an (R) or an (S) configuration. In some embodiments, a disclosed pharmaceutical composition or method of use may comprise a compound with an (R) configuration an (S) configuration, or a mixture of (R) and (S) configurations. In some embodiments, R_1 and R_2 are independently selected from fluoro and hydrogen, with the proviso that R_1 and R_2 may not both be hydrogen.

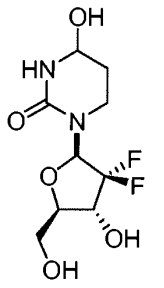
In further embodiments, the compound of Formula I has the stereochemistry of either Ia or Ib:



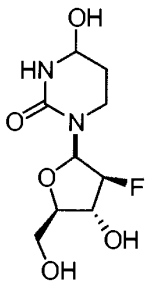
Ia Ib

In some embodiments, R_1 and R_2 are independently selected from fluoro and hydrogen, with the proviso that R_1 and R_2 may not both be hydrogen.

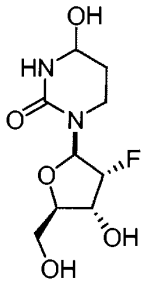
In further embodiments, the compound of Formula I is selected from the group consisting of Compounds 1 to 23 and pharmaceutically acceptable salts thereof:



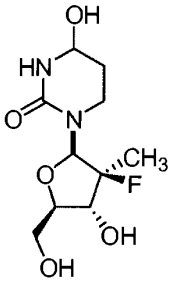
Compound 1



Compound 2



Compound 3



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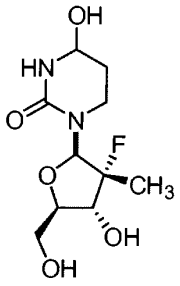

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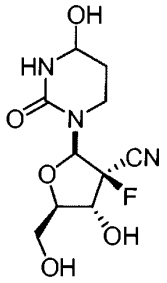

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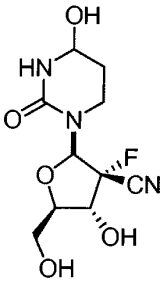

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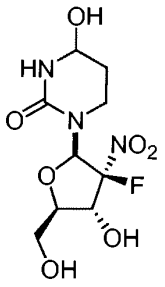

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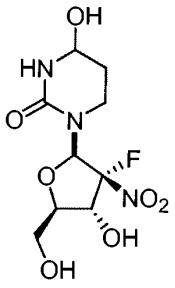
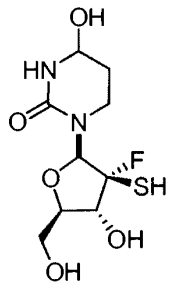


Compound 9



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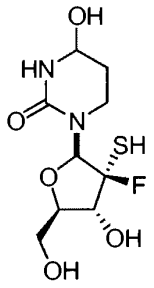

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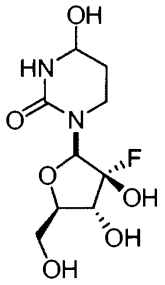

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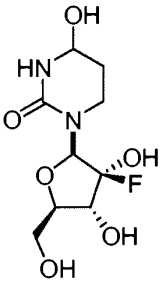
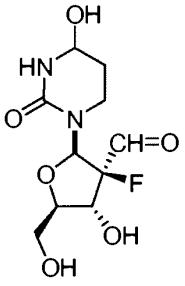


Compound 13



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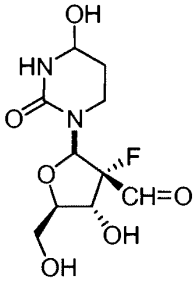

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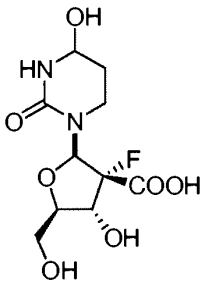
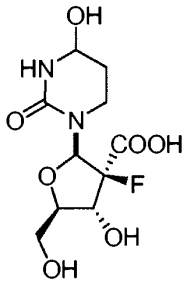
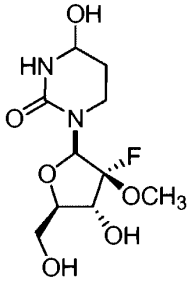


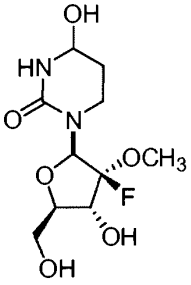
Compound 16



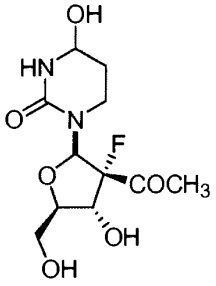
Compound 17



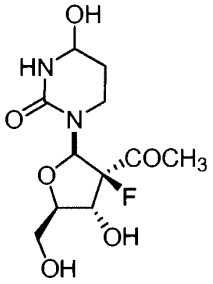
Compound 18



Compound 19



Compound 20



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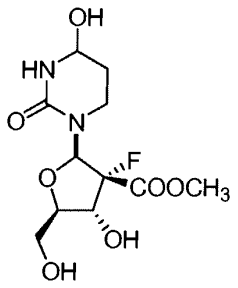
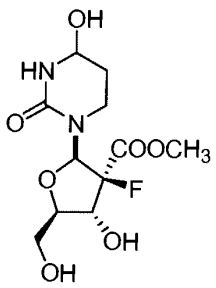
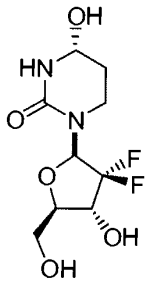


Compound 22

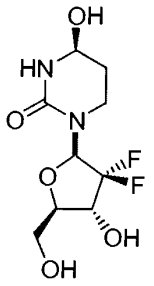


Compound 23

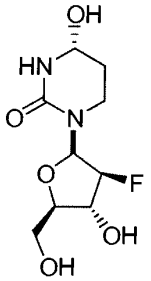
In further embodiments, the compound of Formula I is selected from the group consisting of Compounds 1a, 1b, 2a, 2b, 3a, and 3b, and pharmaceutically acceptable salts thereof.



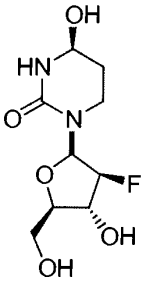
Compound 1a



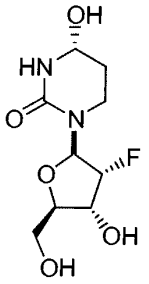
Compound 1b



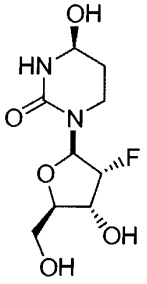
Compound 2a



Compound 2b



Compound 3a



Compound 3b

Since the compounds of the invention may possess at least one chiral center, they may exist in the form of enantiomers, diastereomers, racemic mixtures, non-racemic mixtures or other stereoisomers. The present invention encompasses all such possible isomers, as well as geometric isomers and tautomers.

Another aspect of the present invention relates to a pharmaceutical composition comprising:

(i) an effective amount of a compound of the invention as described herein, including but not limited to each express embodiment; and

(ii) a pharmaceutically acceptable excipient.

In further embodiments, the pharmaceutical composition further comprises an effective amount of at least one additional therapeutic agent, such as a CDA substrate drug or chemotherapeutic agent.

The "effective amount" of the compound of the invention may vary from 0.1 wt. % to about 100 wt. %. In some embodiments, the effective amount of the compound is 0.1 to 20% w/w. In other embodiments, the effective amount is 1-10% w/w. In yet other embodiments, the effective amount is 2-5% w/w.

The pharmaceutical compositions of the invention may be formulated for administration in solid or liquid form, including those adapted for the following: (1) oral administration, for example, drenches (for example, aqueous or non-aqueous solutions or suspensions), tablets (for example, those targeted for buccal, sublingual and systemic absorption), caplets, boluses, powders, granules, pastes for application to the tongue, hard gelatin capsules, soft gelatin capsules, mouth sprays, troches, lozenges, pellets, syrups, suspensions, elixirs, liquids, emulsions and microemulsions; (2) parenteral administration, for example, by subcutaneous, intramuscular, intravenous or epidural injection as, for example, a sterile solution or suspension; (3) topical application, for example, as a cream, ointment, patch, pad or spray applied to the skin; (4) intravaginally or intrarectally, for example, as a pessary, cream or foam; (5) sublingually; (6) ocularly; (7) transdermally; or (8) nasally. The pharmaceutical compositions may be formulated for immediate, sustained or controlled release.

In some embodiments, the pharmaceutical compositions are formulated for oral administration. In further embodiments, the pharmaceutical compositions are formulated for oral administration in solid form.

Another embodiment of the present invention relates to a method for inhibiting cytidine deaminase, comprising administering to a subject in need thereof an effective amount of a compound or pharmaceutical composition of the invention as described herein, including but not limited to each express embodiment.

In some embodiments, the subject is a mammal. In further embodiments, the subject is a human.

Another embodimentof the present invention relates to a method for treating cancer, comprising administering to a subject in need thereof:

(i) an effective amount of a compound or pharmaceutical composition of the invention as described herein, including but not limited to each express embodiment; and

(ii) a CDA substrate drug, including but not limited to each express embodiment described herein.

In some embodiments, the subject is a mammal. In further embodiments, the subject is a human.

In some embodiments, the cancer is selected from hematological cancers and solid cancers. In further embodiments, the hematological cancer selected from MDS and leukemia. In further embodiments, the solid cancer is selected from pancreatic cancer, ovarian cancer, peritoneal cancer, non small cell lung cancer, and breast cancer. In yet further embodiments, the leukemia is acute myeloid leukemia (AML) or chronic myeloid leukemia (CML).

Another embodiment of the present invention relates to a method for inhibiting degradation of a CDA substrate drug by cytidine deaminase, comprising administering an effective amount of a compound or pharmaceutical composition of the invention as described herein, including but not limited to each express embodiment, to a subject that is undergoing treatment with the CDA substrate drug. The CDA substrate drug, including but not limited to each express embodiment described herein.

In some embodiments, the subject is a mammal. In further embodiments, the subject is a human.

Another embodiment of the present invention relates to a kit comprising at least one unit dosage form, which unit dosage form comprises a compound or pharmaceutical composition of the invention.

The kit may further comprise a container and/or a package suitable for commercial sale. The container can be in any conventional shape or form as known in the art which is made of a pharmaceutically acceptable material, such as a paper or cardboard box, a glass or plastic bottle or jar, a re-sealable bag, or a blister pack with individual dosages for pressing out of the pack according to a therapeutic schedule. More than one container can be used together in a single package. For example, tablets may be contained in a blister pack which is in turn contained within a box.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a graph that shows the effect of Compound 1 on decitabine induced survival in the L1210 mouse lymphoma model.

FIG. 2 is a graph that shows the effect of Compound 1a on decitabine induced survival in the L1210 mouse lymphoma model.

FIG. 3 is a graph that shows the effect of Compound 3a on decitabine induced survival in the L1210 mouse lymphoma model.

FIG. 4 is a graph that shows the effect of Compound 1 on Ara-C (200 mg/kg) induced survival in the L1210 model.

FIG. 5 is a graph that shows the effect of Compound 1 on Ara-C (100 mg/kg) induced survival in the L1210 model.

FIG. 6 is a graph that shows the effect of Compound 1 on Ara-C (50 mg/kg) induced survival in the L1210 model.

FIG. 7 is a graph that shows the effect of Compound 1 on Ara-C (25 mg/kg) induced survival in the L1210 model.

FIG. 8 is a graph that shows the effect of Compound 1 on gemcitabine-induced reduction of tumor volume in the mouse A2780 human ovarian cancer xenograft model.

FIG. 9 is an ORTEP plot of the crystal structure of Compound 1a.

FIG. 10 is the ^{1}H NMR structure of THU in D_20.

FIG. 11 is the ^{1}H NMR structure of THU in D_20, in the presence of trifluoroacetic acid, at different times.

FIG. 12 is the ^{1}H NMR structure of Compound 1a in D_20.

FIG. 13 is the ^{1}H NMR structure of Compound 1a in D_20, in the presence of trifluoroacetic acid, at different times.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides certain tetrahydrouridine derivative compounds, pharmaceutical compositions and kits comprising such compounds, and methods of making and using such compounds. The compounds, compositions, kits and methods of the invention may provide certain benefits. For example, the compounds and compositions of the invention may inhibit CDA enzyme activity and/or enhance the half-life, bioavailability and/or efficacy of drugs that are substrates for CDA. Additionally, the compounds, compositions, kits and methods of the invention may exhibit improved aqueous solubility, chemical stability, drug absorption levels, toxicity levels, shelf-life, reproducibility in manufacturing and formulation, and therapeutic efficacy.

Definitions

Throughout the specification and claims, the following definitions apply.

As used in the specification and claims, the singular forms "a," "an," and "the" include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to a pharmaceutical composition comprising "a compound" may encompass two or more compounds.

"Alkyl" refers to a saturated straight or branched chain hydrocarbon radical. Examples include without limitation methyl, ethyl, propyl, iso-propyl, butyl, iso-butyl, *tert*-butyl, n-pentyl and n-hexyl. In some embodiments, the alkyl chain is a C_1 to C_6 branched or unbranched carbon chain. In some embodiments, the alkyl chain is a C_2 to C_5 branched or unbranched carbon chain. In some embodiments, the alkyl chain is a C_1 to C_4 branched or unbranched carbon chain. In some embodiments, the alkyl chain is a C_2 to C_4 branched or unbranched carbon chain. In some embodiments, the alkyl chain is a C_3 to C_5 branched or unbranched carbon chain. In some embodiments, the alkyl chain is a C_1 to C_2 branched or unbranched carbon chain. In some embodiments, the alkyl chain is a C_2 to C_3 branched or unbranched carbon chain.

"Alkenyl" refers to an unsaturated straight or branched chain hydrocarbon radical comprising at least one carbon to carbon double bond. Examples include without limitation ethenyl, propenyl, iso-propenyl, butenyl, iso-butenyl, *tert*-butenyl, n-pentenyl and n-hexenyl. In some embodiments, the alkenyl chain is a C_2 to C_6 branched or unbranched carbon chain. In some embodiments, the alkenyl chain is a C_2 to C_5 branched or unbranched carbon chain. In some embodiments, the alkenyl chain is a C_2 to C_4 branched or unbranched carbon chain. In some embodiments, the alkenyl chain is a C_3 to C_5 branched or unbranched carbon chain.

"Alkoxy" refers to an alkyl group bonded through an oxygen linkage.

"Alkenoxy" refers to an alkenyl group bonded through an oxygen linkage.

"Cycloalkyl" refers to a non-aromatic cyclic alkyl radical.

"Cycloalkenyl" refers to a non-aromatic cyclic alkenyl radical.

"Halo" refers to a fluoro, chloro, bromo or iodo radical.

"Substituted" means that at least one hydrogen on a designated group is replaced with another radical, provided that the designated group's normal valence is not exceeded. With respect to any group containing one or more substituents, such groups are not intended to introduce any substitution that is sterically impractical, synthetically non-feasible and/or inherently unstable.

"CDA substrate drug" refers to a drug that can be deaminated by CDA. Nonlimiting examples of a CDA substrate include cytidine analogs, such as decitabine, 5-azacytidine, gemcitabine, ara-C, troxacitabine, tezacitabine, 5'-fluoro-2'-deoxycytidine, and cytochlor.

"Effective amount" refers to the amount required to produce a desired effect (e.g., enhancing the half-life, bioavailability or efficacy of a CDA substrate drug, treating cancer in a subject, inhibiting cytidine deaminase in a subject, or inhibiting degradation of a CDA substrate drug by cytidine deaminase).

"Half-life" refers to the period of time required for the concentration or amount of a compound in a subject to be reduced to exactly one-half of a given concentration or amount.

"Pharmaceutically acceptable" refers to those properties and/or substances that are acceptable to the patient from a pharmacological and/or toxicological point of view, and/or to the manufacturing pharmaceutical chemist from a physical and/or chemical point of view regarding composition, formulation, stability, patient acceptance, bioavailability and compatibility with other ingredients.

"Pharmaceutically acceptable excipient" can mean any substance, not itself a therapeutic agent, used as a carrier, diluent, adjuvant, binder, and/or vehicle for delivery of a therapeutic agent to a subject, or added to a pharmaceutical composition to improve its handling or storage properties or to permit or facilitate formation of a compound or composition into a unit dosage form for administration. Pharmaceutically acceptable excipients are well known in the pharmaceutical arts and are described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, Pa (e.g., 20th Ed., 2000), and Handbook of Pharmaceutical Excipients, American Pharmaceutical Association, Washington, D.C., (e.g., 1st, 2nd and 3rd Eds., 1986, 1994 and 2000, respectively). As will be known to those skilled in the art, excipients may provide a variety of functions and may be described as wetting agents, buffering agents, suspending agents, lubricating agents, emulsifiers, disintegrants, absorbents, preservatives, surfactants, colorants, flavorants, and sweeteners. Examples of pharmaceutically acceptable excipients include without limitation: (1) sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose, cellulose acetate, hydroxypropylmethylcellulose, and hydroxypropylcellulose; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols, such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; (12) esters, such as ethyl oleate and ethyl

laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; (20) pH buffered solutions; (21) polyesters, polycarbonates and/or polyanhydrides; and (22) other non-toxic compatible substances employed in pharmaceutical formulations.

"Pharmaceutically acceptable salt" refers to an acid or base salt of a compound of the invention, which salt possesses the desired pharmacological activity and is neither biologically nor otherwise undesirable. The salt can be formed with acids that include without limitation acetate, adipate, alginate, aspartate, benzoate, benzenesulfonate, bisulfate butyrate, citrate, camphorate, camphorsulfonate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, fumarate, glucoheptanoate, glycerophosphate, hemisulfate, heptanoate, hexanoate, hydrochloride hydrobromide, hydroiodide, 2-hydroxyethane-sulfonate, lactate, maleate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, oxalate, thiocyanate, tosylate and undecanoate. Examples of a base salt include without limitation ammonium salts, alkali metal salts such as sodium and potassium salts, alkaline earth metal salts such as calcium and magnesium salts, salts with organic bases such as dicyclohexylamine salts, N-methyl-D-glucamine, and salts with amino acids such as arginine and lysine. In some embodiments, the basic nitrogen-containing groups can be quarternized with agents including lower alkyl halides such as methyl, ethyl, propyl and butyl chlorides, bromides and iodides; dialkyl sulfates such as dimethyl, diethyl, dibutyl and diamyl sulfates; long chain halides such as decyl, lauryl, myristyl and stearyl chlorides, bromides and iodides; and aralkyl halides such as phenethyl bromides.

"Unit dosage form" refers to a physically discrete unit suitable as a unitary dosage for human or other animal subjects. Each unit dosage form may contain a predetermined amount of an active substance (e.g., compound or composition of the invention, CDA substrate drug and/or other therapeutic agent) calculated to produce a desired effect.

"Isomers" refer to compounds having the same number and kind of atoms and hence the same molecular weight, but differing with respect to the arrangement or configuration of the atoms.

"Stereoisomers" refer to isomers that differ only in the arrangement of the atoms in space.

"Diastereoisomers" refer to stereoisomers that are not mirror images of each other.

"Enantiomers" refers to stereoisomers that are non-superimposable mirror images of one another. Enantiomers include "enantiomerically pure" isomers that comprise substantially

a single enantiomer, for example, greater than or equal to 90%, 92%, 95%, 98%, or 99%, or equal to 100% of a single enantiomer.

"Epimers" refer to stereoisomers of a compound that have different configurations at only one of several stereogenic centers.

"Racemic" refers to a mixture containing equal parts of individual enantiomers.

"Non-racemic" refers to a mixture containing unequal parts of individual enantiomers. A non-racemic mixture may be enriched in the R- or S-configuration, including, without limitation, about 50/50, about 60/40, and about 70/30 R- to S-enantiomer, or S- to R-enantiomer, mixtures.

"Optional" or "optionally" means that the subsequently described event or circumstance may or may not occur, and that the description includes instances where the event or circumstance occurs and instances in which it does not. For example, an alkyl that is "optionally substituted" encompasses both an alkyl that is unsubstituted and an alkyl that is substituted.

"Subject" refers to a cell or tissue, *in vitro* or *in vivo*, an animal or a human. An animal or human subject may also be referred to as a "patient."

"Animal" refers to a living organism having sensation and the power of voluntary movement, and which requires for its existence oxygen and organic food.

"Mammal" refers to a warm-blooded vertebrate animal with hair or fur. Examples include without limitation members of the human, equine, porcine, bovine, murine, canine or feline species.

"Treating" in reference to a disease, disorder or condition refers to: (i) inhibiting a disease, disorder or condition, e.g., arresting its development; and/or (ii) relieving a disease, disorder or condition, e.g., causing regression of the clinical symptoms.

"Preventing" in reference to a disease, disorder or condition refers to preventing a disease, disorder or condition, e.g., causing the clinical symptoms of the disease, disorder or condition not to develop.

"Cancer" refers to an abnormal growth of cells which tend to proliferate in an uncontrolled way and, in some cases, to metastasize (spread). Specific cancers types include without limitation the cancers identified in Publication No. US 2006/0014949 and the following:

- cardiac: sarcoma (e.g., such as angiosarcoma, fibrosarcoma, rhabdomyosarcoma, liposarcoma and the like), myxoma, rhabdomyoma, fibroma, lipoma and teratoma;

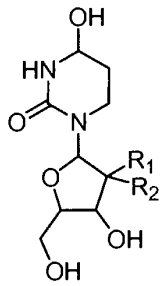
- lung: bronchogenic carcinoma (e.g., such as squamous cell, undifferentiated small cell, undifferentiated large cell, adenocarcinoma and the like), alveolar (e.g., such as bronchiolar) carcinoma, bronchial adenoma, sarcoma, lymphoma, chondromatous hamartoma, mesothelioma;
- gastrointestinal: esophagus (e.g., such as squamous cell carcinoma, adenocarcinoma, leiomyosarcoma, lymphoma and the like), stomach (e.g., such as carcinoma, lymphoma, leiomyosarcoma and the like), pancreas (e.g., such as ductal adenocarcinoma, insulinoma, glucagonoma, gastrinoma, carcinoid tumors, vipoma and the like), small bowel (e.g., such as adenocarcinoma, lymphoma, carcinoid tumors, Karposi's sarcoma, leiomyoma, hemangioma, lipoma, neurofibroma, fibroma, and the like), large bowel (e.g., such as adenocarcinoma, tubular adenoma, villous adenoma, hamartoma, leiomyoma and the like);
- genitourinary tract: kidney (e.g., such as adenocarcinoma, Wilm's tumor nephroblastoma, lymphoma, leukemia, and the like), bladder and urethra (e.g., such as squamous cell carcinoma, transitional cell carcinoma, adenocarcinoma and the like), prostate (e.g., such as adenocarcinoma, sarcoma), testis (e.g., such as seminoma, teratoma, embryonal carcinoma, teratocarcinoma, choriocarcinoma, sarcoma, interstitial cell carcinoma, fibroma, fibroadenoma, adenomatoid tumors, lipoma and the like);
- liver: hepatoma (e.g., hepatocellular carcinoma and the like), cholangiocarcinoma, hepatoblastoma, angiosarcoma, hepatocellular adenoma, hemangioma;
- bone: osteogenic sarcoma (e.g., such as osteosarcoma and the like), fibrosarcoma, malignant fibrous histiocytoma, chondrosarcoma, Ewing's sarcoma, malignant lymphoma (e.g., such as reticulum cell sarcoma), multiple myeloma, malignant giant cell tumor chordoma, osteochronfroma (e.g., such as osteocartilaginous exostoses), benign chondroma, chondroblastoma, chondromyxofibroma, osteoid osteoma and giant cell tumors;
- nervous system: skull (e.g., such as osteoma, hemangioma, granuloma, xanthoma, osteitis deformans and the like), meninges (e.g., such as meningioma, meningiosarcoma, gliomatosis and the like), brain (e.g., such as astrocytoma, medulloblastoma, glioma, ependymoma, germinoma [pinealoma], glioblastoma multiform, oligodendroglioma, schwannoma, retinoblastoma,

congenital tumors and the like), spinal cord (e.g., such as neurofibroma, meningioma, glioma, sarcoma and the like);

- gynecological: uterus (e.g., such as endometrial carcinoma and the like), cervix (e.g., such as cervical carcinoma, pre-tumor cervical dysplasia and the like), ovaries (e.g., such as ovarian carcinoma [serous cystadenocarcinoma, mucinous cystadenocarcinoma, unclassified carcinoma], granulosa-thecal cell tumors, Sertoli-Leydig cell tumors, dysgerminoma, malignant teratoma, and the like), vulva (e.g., such as squamous cell carcinoma, intraepithelial carcinoma, adenocarcinoma, fibrosarcoma, melanoma and the like), vagina (e.g., such as clear cell carcinoma, squamous cell carcinoma, botryoid sarcoma (embryonal rhabdomyosarcoma], fallopian tubes (carcinoma) and the like);
- hematologic: blood (e.g., such as myeloid leukemia [acute and chronic], acute lymphoblastic leukemia, chronic lymphocytic leukemia, myeloproliferative diseases, multiple myeloma, myelodysplastic syndrome and the like), Hodgkin's disease, non-Hodgkin's lymphoma;
- skin: malignant melanoma, basal cell carcinoma, squamous cell carcinoma, Karposi's sarcoma, moles dysplastic nevi, lipoma, angioma, dermatofibroma, keloids, psoriasis and the like; and
- adrenal glands: neuroblastoma.

Compounds

One aspect of the present invention relates to a compound of Formula I:



I

or a pharmaceutically acceptable salt of the compound, wherein:

R_1 and R_2 are independently selected from the group consisting of hydrogen, halo, cyano, nitro, sulfhydryl, hydroxyl, formyl, carboxyl, $COO(C_1$ to C_6 straight or branched chain alkyl), $COO(C_1$ to C_6 straight or branched chain alkenyl), $COO(C_1$ to C_6 straight or branched chain alkynyl), $CO(C_1$ to C_6 straight or branched chain alkyl), $CO(C_1$ to C_6 straight or branched

chain alkenyl), CO(C_1 to C_6 straight or branched chain alkynyl), C_1 to C_6 straight or branched chain alkyl, C_1 to C_6 straight or branched chain alkenyl, C_1 to C_6 straight or branched chain alkynyl, C_1 to C_6 straight or branched chain alkoxy, and C_1 to C_6 straight or branched chain alkenoxy; wherein each occurrence of C_1 to C_6 straight or branched chain alkyl, C_1 to C_6 straight or branched chain alkenyl, C_1 to C_6 straight or branched chain alkynyl, C_1 to C_6 straight or branched chain alkoxy, or C_1 to C_6 straight or branched chain alkenoxy may be independently unsubstituted or substituted with one to four substituents independently selected from the group consisting of halo, hydroxyl, cyano, nitro, formyl, carboxyl, and sulfhydryl;

and provided that when one of R_1 and R_2 is -H, then the other is not -H, -OH or $-CH_2OH$.

In some embodiments, R_1 and R_2 are independently selected from the group consisting of hydrogen, halo, hydroxyl, cyano, nitro, sulfhydryl, C_1 to C_6 straight or branched chain alkyl, C_1 to C_6 straight or branched chain alkenyl, C_1 to C_6 straight or branched chain alkynyl, C_1 to C_6 straight or branched chain alkoxy, and C_1 to C_6 straight or branched chain alkenoxy; wherein wherein each occurrence of C_1 to C_6 straight or branched chain alkyl, C_1 to C_6 straight or branched chain alkenyl, C_1 to C_6 straight or branched chain alkynyl, C_1 to C_6 straight or branched chain alkoxy, or C_1 to C_6 straight or branched chain alkenoxy may be independently unsubstituted or substituted with one ore more halos;

and provided that when one of R_1 and R_2 is -H, then the other is not -H, or -OH.

In some embodiments, R_1 and R_2 are independently selected from the group consisting of hydrogen, halo, C_1 to C_6 alkyl, C_1 to C_6 alkenyl, C_1 to C_6 alkoxy, and C_1 to C_6 alkenoxy; wherein each occurrence of C_1 to C_6 straight or branched chain alkyl, C_1 to C_6 straight or branched chain alkenyl, C_1 to C_6 straight or branched chain alkoxy and C_1 to C_6 straight or branched chain alkenoxy may be independently unsubstituted or substituted with one to three halos;

and provided that when one of R_1 and R_2 is -H, then the other is not -H, or -OH.

In further embodiments, at least one of R_1 and R_2 is halo.

In further embodiments, at least one of R_1 and R_2 is fluoro.

In further embodiments, one of R_1 and R_2 is halo, and the other is -H.

In further embodiments, one of R_1 and R_2 is fluoro, and the other is -H.

In further embodiments, R_1 and R_2 are each fluoro.

In further embodiments, one of R_1 and R_2 is halo, and the other is -CN.

In further embodiments, one of R_1 and R_2 is fluoro, and the other is -CN.

In further embodiments, one of R_1 and R_2 is halo, and the other is $-NO_2$.

In further embodiments, one of R_1 and R_2 is fluoro, and the other is $-NO_2$.

In further embodiments, one of R_1 and R_2 is halo, and the other is -SH.

In further embodiments, one of R_1 and R_2 is fluoro, and the other is -SH.

In further embodiments, one of R_1 and R_2 is halo, and the other is -OH.

In further embodiments, one of R_1 and R_2 is fluoro, and the other is -OH.

In further embodiments, one of R_1 and R_2 is halo, and the other is -CHO.

In further embodiments, one of R_1 and R_2 is fluoro, and the other is -CHO.

In further embodiments, one of R_1 and R_2 is halo, and the other is -COOH.

In further embodiments, one of R_1 and R_2 is fluoro, and the other is -COOH.

In further embodiments, one of R_1 and R_2 is halo, and the other is $-COOR_x$, wherein R_x selected from the group consisting of C_1 to C_6 straight or branched chain alkyl, C_1 to C_6 straight or branched chain alkenyl, and C_1 to C_6 straight or branched chain alkynyl.

In further embodiments, one of R_1 and R_2 is fluoro, and the other is $-COOR_x$, wherein R_x selected from the group consisting of C_1 to C_6 straight or branched chain alkyl, C_1 to C_6 straight or branched chain alkenyl, and C_1 to C_6 straight or branched chain alkynyl.

In further embodiments, one of R_1 and R_2 is halo, and the other is $-COR_x$, wherein R_x selected from the group consisting of C_1 to C_6 straight or branched chain alkyl, C_1 to C_6 straight or branched chain alkenyl, and C_1 to C_6 straight or branched chain alkynyl.

In further embodiments, one of R_1 and R_2 is fluoro, and the other is $-COR_x$, wherein R_x selected from the group consisting of C_1 to C_6 straight or branched chain alkyl, C_1 to C_6 straight or branched chain alkenyl, and C_1 to C_6 straight or branched chain alkynyl.

In further embodiments, one of R_1 and R_2 is halo, and the other is $-C_1$ to C_6 straight or branched chain alkyl.

In further embodiments, one of R_1 and R_2 is fluoro, and the other is $-C_1$ to C_6 straight or branched chain alkyl.

In further embodiments, one of R_1 and R_2 is halo, and the other is $-C_1$ to C_6 straight or branched chain alkenyl.

In further embodiments, one of R_1 and R_2 is fluoro, and the other is $-C_1$ to C_6 straight or branched chain alkenyl.

In further embodiments, one of R_1 and R_2 is halo, and the other is $-C_1$ to C_6 straight or branched chain alkoxy.

In further embodiments, one of R_1 and R_2 is fluoro, and the other is $-C_1$ to C_6 straight or branched chain alkoxy.

In further embodiments, one of R_1 and R_2 is halo, and the other is -C_1 to C_6 straight or branched chain alkenoxy.

In further embodiments, one of R_1 and R_2 is fluoro, and the other is -C_1 to C_6 straight or branched chain alkenoxy.

In further embodiments, at least one of R_1 and R_2 is -C_1 to C_6 straight or branched chain alkyl substituted with halo.

In further embodiments, one of R_1 and R_2 is -C_1 to C_6 straight or branched chain alkyl substituted with halo, and the other is -H.

In further embodiments, one of R_1 and R_2 is -C_1 to C_6 straight or branched chain alkyl substituted with fluoro, and the other is -H.

In further embodiments, at least one of R_1 and R_2 is -C_1 to C_6 straight or branched chain alkenyl substituted with halo.

In further embodiments, one of R_1 and R_2 is -C_1 to C_6 straight or branched chain alkenyl substituted with halo, and the other is -H.

In further embodiments, one of R_1 and R_2 is -C_1 to C_6 straight or branched chain alkenyl substituted with fluoro, and the other is -H.

In further embodiments, at least one of R_1 and R_2 is/are -C_1 to C_6 straight or branched chain alkoxy substituted with halo

In further embodiments, one of R_1 and R_2 is -C_1 to C_6 alkoxy substituted with halo, and the other is -H.

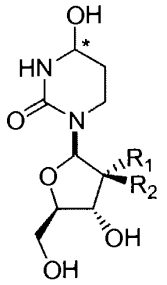
In further embodiments, one of R_1 and R_2 is -C_1 to C_6 straight or branched chain alkoxy substituted with fluoro, and the other is -H.

In further embodiments, at least one of R_1 and R_2 is -C_1 to C_6 straight or branched chain alkenoxy substituted with halo.

In further embodiments, one of R_1 and R_2 is -C_1 to C_6 straight or branched chain alkenoxy substituted with halo, and the other is -H.

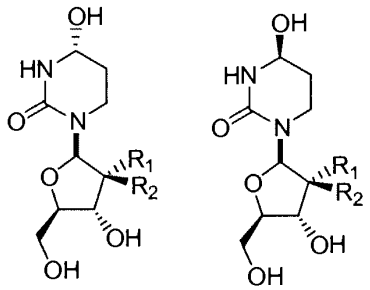
In further embodiments, one of R_1 and R_2 is -C_1 to C_6 straight or branched chain alkenoxy substituted with fluoro, and the other is -H.

In some embodiments, the compound of Formula is:



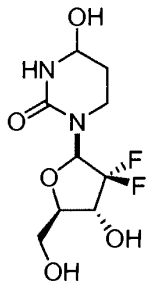
wherein the carbon marked by an asterisk may have an (R) or an (S) configuration. In some embodiments, a disclosed pharmaceutical composition or method of use may comprise a compound with an (R) configuration an (S) configuration, or a mixture of (R) and (S) configurations. In some embodiments, R_1 and R_2 are independently selected from fluoro and hydrogen, with the proviso that R_1 and R_2 may not both be hydrogen.

In further embodiments, the compound of Formula I has the stereochemistry of either Ia or Ib:

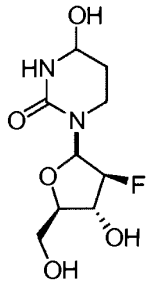


Ia Ib

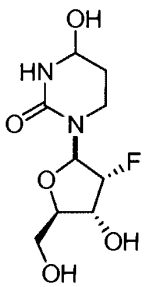
In further embodiments, the compound of Formula I is selected from the group consisting of Compounds 1 to 23 and pharmaceutically acceptable salts thereof:



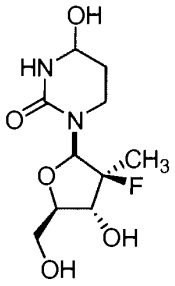
Compound 1



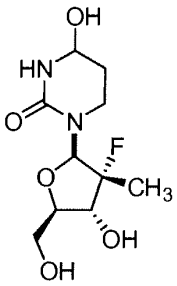
Compound 2



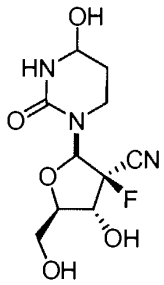
Compound 3



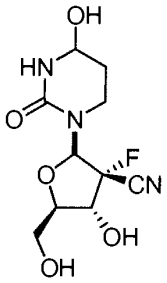
Compound 4



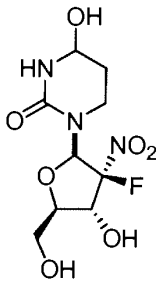
Compound 5



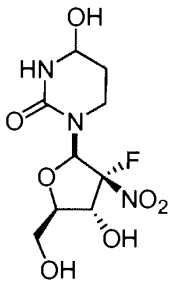
Compound 6



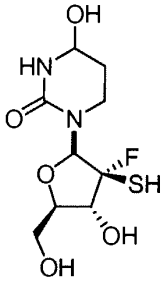
Compound 7



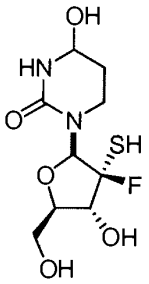
Compound 8



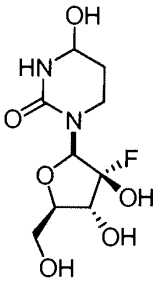
Compound 9



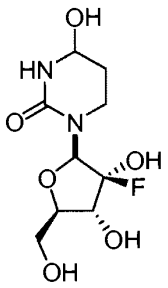
Compound 10



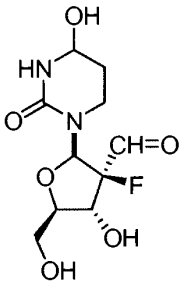
Compound 11



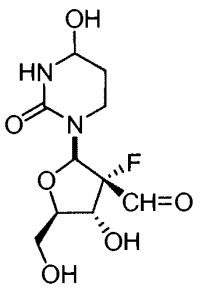
Compound 12



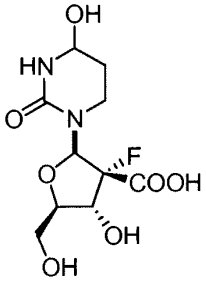
Compound 13



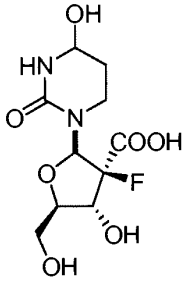
Compound 14



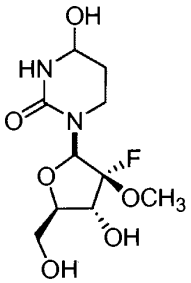
Compound 15



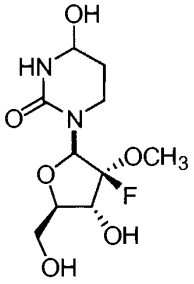
Compound 16



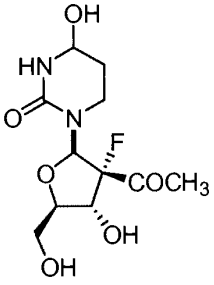
Compound 17



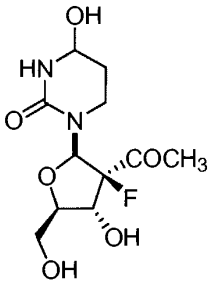
Compound 18



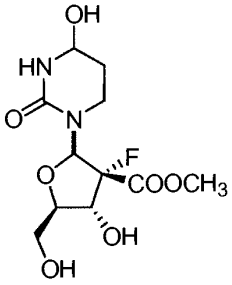
Compound 19



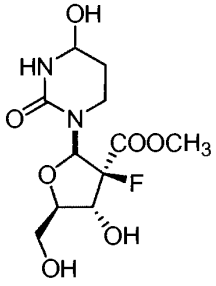
Compound 20



Compound 21

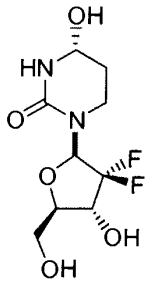


Compound 22

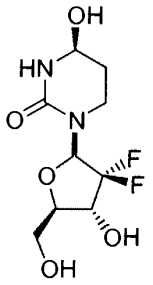


Compound 23

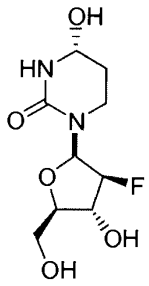
In further embodiments, the compound of Formula I is selected from the group consisting of Compounds 1a, 1b, 2a, 2b, 3a, 3b, and pharmaceutically acceptable salts thereof.



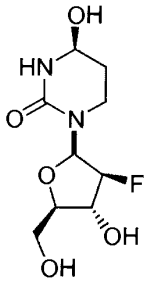
Compound 1a



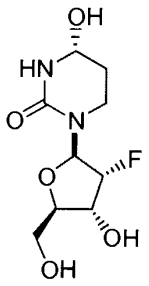
Compound 1b



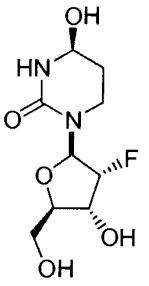
Compound 2a



Compound 2b



Compound 3a



Compound 3b

Since the compounds of the invention may possess at least one chiral center, they may exist in the form of enantiomers, diastereomers, racemic mixtures, non-racemic mixtures or other stereoisomers. The present invention encompasses all such possible isomers, as well as geometric isomers and tautomers.

Stereoisomers may be prepared or isolated by known methods. For example, diastereoisomers may be separated by physical separation methods such as fractional crystallization and chromatographic techniques, and enantiomers may be separated from each other by the selective crystallization of the diastereomeric salts with optically active acids or bases or by chiral chromatography. Pure stereoisomers may also be prepared synthetically from appropriate stereochemically pure starting materials, or by using stereoselective reactions.

Pharmaceutical Compositions

Another aspect of the present invention relates to a pharmaceutical composition comprising:

(i) an effective amount of a compound of the invention as described herein, including but not limited to each express embodiment; and

(ii) a pharmaceutically acceptable excipient.

In further embodiments, the pharmaceutical composition further comprises an effective amount of at least one additional therapeutic agent, such as a CDA substrate drug or chemotherapeutic agent.

The CDA substrate drug may be any drug that can be deaminated by CDA. Nonlimiting examples of a CDA substrate include cytosine and cytidine analogs, such as decitabine, 5-azacytidine, gemcitabine, ara-C, troxacitabine, tezacitabine, 5'-fluoro-2'-deoxycytidine, cytochlor, and the compounds disclosed in US Publication No. 2006/0014949. In some embodiments, the CDA substrate drug is decitabine. In other embodiments, the CDA substrate drug is 5-azacytidine. In yet other embodiments, the CDA substrate drug is gemcitabine. In yet other embodiments, the CDA substrate drug is ara-C.

Examples of a chemotherapeutic agent include without limitation:

alkylating agents (e.g., which may include doxorubicin, cyclophosphamide, estramustine, carmustine, mitomycin, bleomycin and the like);

antimetabolites (e.g., which may include 5-Fluoro-Uracil, capecitabine, gemcitabine, nelarabine, fludarabine, methotrexate and the like);

platinating agents (e.g., which may include cisplatin, oxaliplatin, carboplatin and the like);

topoisomerase inhibitors (e.g., which may include topotecan, irinotecan, etoposide and the like);

tubulin agents (e.g., which may include paclitaxel, docetaxel, vinorelbine, vinblastine, vincristine, other taxanes, epothilones, and the like);

signalling inhibitors (e.g., kinase inhibitors, antibodies, farnesyltransferase inhibitors, and the like); and

other chemotherapeutic agents (e.g, tamoxifen, anti-mitotic agents such as polo-like kinase inhibitors or aurora kinase inhibitors, and the like).

The "effective amount" of the compound of the invention may vary from 0.1 wt. % to about 100 wt. %. In some embodiments, the effective amount of the compound is 0.1 to 20% w/w. In other embodiments, the effective amount is 1-10% w/w. In yet other embodiments, the effective amount is 2-5% w/w.

The pharmaceutical compositions of the invention may be formulated for administration in solid or liquid form, including those adapted for the following: (1) oral

administration, for example, drenches (for example, aqueous or non-aqueous solutions or suspensions), tablets (for example, those targeted for buccal, sublingual and systemic absorption), caplets, boluses, powders, granules, pastes for application to the tongue, hard gelatin capsules, soft gelatin capsules, mouth sprays, troches, lozenges, pellets, syrups, suspensions, elixirs, liquids, emulsions and microemulsions; (2) parenteral administration, for example, by subcutaneous, intramuscular, intravenous or epidural injection as, for example, a sterile solution or suspension; (3) topical application, for example, as a cream, ointment, patch, pad or spray applied to the skin; (4) intravaginally or intrarectally, for example, as a pessary, cream or foam; (5) sublingually; (6) ocularly; (7) transdermally; or (8) nasally. The pharmaceutical compositions may be formulated for immediate, sustained or controlled release.

In some embodiments, the pharmaceutical compositions are formulated for oral administration. In further embodiments, the pharmaceutical compositions are formulated for oral administration in solid form.

Pharmaceutical compositions of the invention can be prepared using known materials and techniques, which may include but are not limited to mixing and/or blending the compound of the invention with the pharmaceutically acceptable excipient and optional therapeutic agent(s).

Methods

Another aspect of the present invention relates to a method for inhibiting cytidine deaminase, comprising administering to a subject in need thereof an effective amount of a compound or pharmaceutical composition of the invention as described herein, including but not limited to each express embodiment.

In some embodiments, the subject is a mammal. In further embodiments, the subject is a human.

Another aspect of the present invention relates to a method for treating cancer, comprising administering to a subject in need thereof:

(i) an effective amount of a compound or pharmaceutical composition of the invention as described herein, including but not limited to each express embodiment; and

(ii) a CDA substrate drug, including but not limited to each express embodiment described herein.

In some embodiments, the subject is a mammal. In further embodiments, the subject is a human.

In some embodiments, the cancer is selected from hematological cancers and solid cancers. In further embodiments, the hematological cancer selected from MDS and leukemia. In further embodiments, the solid cancer is selected from pancreatic cancer, ovarian cancer, peritoneal cancer, non small cell lung cancer, and breast cancer. In yet further embodiments, the leukemia is acute myeloid leukemia (AML) or chronic myeloid leukemia (CML).

Another aspect of the present invention relates to a method for inhibiting degradation of a CDA substrate drug by cytidine deaminase, comprising administering an effective amount of a compound or pharmaceutical composition of the invention as described herein, including but not limited to each express embodiment, to a subject that is undergoing treatment with the CDA substrate drug. The CDA substrate drug, including but not limited to each express embodiment described herein.

In some embodiments, the subject is a mammal. In further embodiments, the subject is a human.

Administration of the compound or composition of the invention may be via any accepted mode known to one skilled in the art, for example, orally, parenterally, by inhalation spray, topically, rectally, nasally, buccally, vaginally, intraoccularly, intrapulmonarily, or via an implanted reservoir. The term "parenterally" includes without limitation subcutaneously, intravenously, intramuscularly, intraperitoneally, intrathecally, intraventricularly, intrasternally, intracranially, by intraosseous injection and by infusion techniques.

Any administration regimen well known to those skilled in the art for regulating the timing and sequence of drug delivery can be used and repeated as necessary to effect treatment in the methods of the invention. For example, the compound or composition of the invention may be administered 1, 2, 3 or 4 times daily, by a single dose, multiple discrete doses or continuous infusion.

The compound or composition of the invention may be administered prior to, at substantially the same time with, or after administration of the CDA substrate drug. The administration regimen may include pretreatment and/or co-administration with at least one additional therapeutic agent. In such case, the compound or composition of the invention, CDA substrate drug and at least one additional therapeutic agent may be administered simultaneously, separately, or sequentially.

Examples of administration regimens include without limitation:

administration of each compound, composition, CDA substrate drug, and/or therapeutic agent in a sequential manner; and

co-administration of each compound, composition, CDA substrate drug, and/or therapeutic agent in a substantially simultaneous manner (e.g., as in a single unit dosage form) or in multiple, separate unit dosage forms for each compound, composition, CDA substrate drug, and/or therapeutic agent.

It will be appreciated by those skilled in the art that the "effective amount" or "dose level" will depend on various factors such as the particular administration mode, administration regimen, compound, and composition selected, and the particular disease and patient being treated. For example, the appropriate dose level may vary depending upon the activity, rate of excretion and possible toxicity of the specific compound or composition employed; the age, body weight, general health, gender and diet of the patient being treated; the frequency of administration; the other therapeutic agent(s) being co-administered; and the type and severity of the disease.

The present invention contemplates dose levels on the order of about 0.001 to about 10,000 mg/kg/d. In some embodiments, the dose level is about 0.1 to about 1,000 mg/kg/d. In other embodiments, the dose level is about 1 to about 100 mg/kg/d. In yet other embodiments, the dose level is about 1 to about 50 mg/kg/d. In yet other embodiments, the dose level is about 1 to about 25 mg/kg/d. Appropriate dose levels, mode of administration, and administration regimen may be ascertained by those skilled in the art using known techniques.

Kits

Another aspect of the present invention relates to a kit comprising at least one unit dosage form, which unit dosage form comprises a compound or pharmaceutical composition of the invention.

The kit may further comprise a container and/or a package suitable for commercial sale. The container can be in any conventional shape or form as known in the art which is made of a pharmaceutically acceptable material, such as a paper or cardboard box, a glass or plastic bottle or jar, a re-sealable bag, or a blister pack with individual dosages for pressing out of the pack according to a therapeutic schedule. More than one container can be used together in a single package. For example, tablets may be contained in a blister pack which is in turn contained within a box.

The kit may further comprise information. The information may be provided on a readable medium. The readable medium may comprise a label. The information may be directed towards a physician, pharmacist or patient. The information may indicate that the unit dosage form may cause one or more adverse effects. The information may comprise

instructions for administering the unit dosage form, such as in a manner described herein. These instructions may be provided in a variety of ways. For example, the information may include a table including a variety of weights or weight ranges and appropriate dosages for each weight or weight range.

The information can be associated with the container, for example, by being: written on a label (e.g., the prescription label or a separate label) adhesively affixed to a container; included inside a container as a written package insert; applied directly to the container such as being printed on the wall of a box or blister pack; or attached as by being tied or taped, for example as an instructional card affixed to the neck of a bottle via a string, cord or other line, lanyard or tether type device.

It will be apparent to those skilled in the art that specific embodiments of the present invention may be directed to one, some or all of the above-indicated aspects as well as other aspects, and may encompass one, some or all of the above- and below- indicated embodiments, as well as other embodiments.

Other than in the working examples, or where otherwise indicated, all numbers expressing quantities of ingredients, reaction conditions, and so forth used in the specification and claims are to be understood as being modified by the term "about". Accordingly, unless indicated to the contrary, such numbers are approximations that may vary depending upon the-desired properties sought to be obtained by the present invention. At the very least, and not as an attempt to limit the application of the doctrine of equivalents to the scope of the claims, each numerical parameter should be construed in light of the number of significant digits and ordinary rounding techniques.

While the numerical ranges and parameters setting forth the broad scope of the invention are approximations, the numerical values set forth in the working examples are reported as precisely as possible. Any numerical value, however, inherently contains certain errors necessarily resulting from the standard deviation found in their respective testing measurements.

EXAMPLES

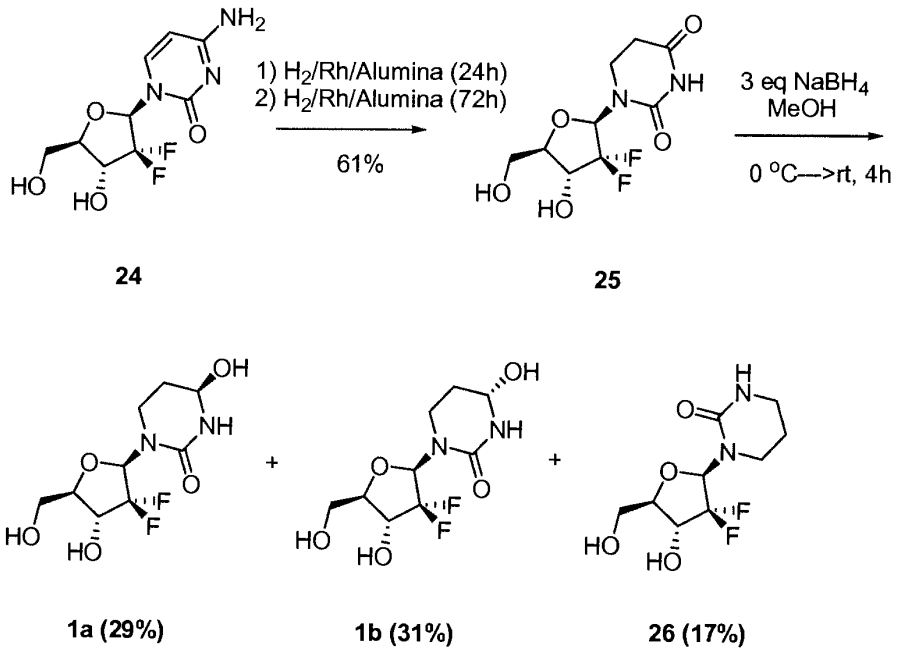
The following examples are illustrative of the present invention and are not intended to be limitations thereon.

Synthesis of Compounds

Compound of the invention can be prepared as described herein and/or by the application or adaptation of known methods. It will be appreciated by those skilled in the art that one or more of the reactants, steps and/or conditions described in the reaction schemes may require adjustment to accommodate other substituents at R_1 and R_2.

Example 1:

Scheme 1. Synthesis of difluorotetrahydrouridine derivatives (Compounds 1a and 1b)



2'2'-DiFluoro-DiHydro-Uridine (DFDHU, 25). Gemcitabine **24** (3.0 g, 11.4 mmol) is dissolved in H_2O (50 mL). Rhodium on alumina (900 mg) is added to the solution and the mixture is hydrogenated overnight at 40 psi. The next day, the mixture is filtered, the water is removed *in vacuo* and the resulting sticky solid is dissolved in H_2O again. Rhodium on alumina is added to the solution (900 mg) and the material is hydrogenated overnight at 40 psi. The rhodium is filtered off and the resulting filtrate is concentrated to afford a crude mixture of difluorodihydrouridine (**5**, DFDHU) and ~10% of difluorotetrahydrouridine **1a** and **1b** (DFTHU). The crude mixture is purified on reverse phase HPLC (reverse phase C_{18} @ 5% CH_3CN/H_2O) to afford 1.84 g (61%, 14.5 minutes) of DFDHU **25** and 175 mg (17%, **1a**, 9.5 minutes and **1b**, 13.9 minutes) of the epimers of DFTHU. The absolute configuration of C-4 for compound 1a is determined by single crystal X-ray diffraction, and is consistent with literature precedents on the crystal structure of cytidine deaminase in complex with a single

epimer of tetrahydrouridine. ^{1}HNMR of **5**: 6.00 (dd, 1H), 4.20 (q, 1H), 3.92-3.72 (m, 3H), 3.64 (m, 1H), 3.43 (m, 1H), 2.68 (t, 2H).

2'2'-DiFluoro-TetraHydroUridine (DFTHU, 1a and 1b). The DFDHU **25** (1.2 g, 4.9 mmol) is dissolved in 30 mL of MeOH and cooled to 0°C. Sodium borohydride (540 mg, 14.6 mmol) is added portion-wise to the solution and the reaction is slowly warmed to room temperature. After 4 hours of stirring at room temperature (r.t.), the MeOH is removed *in vacuo* and the residue is dissolved in 15 mL of H_2O. The solution is neutralized with 2.0 N HCl to pH 7. The solution is then purified via prep HPLC (reverse phase C_{18} @ 5% CH_3CN/H_2O). The salts come out at 5.2 minutes. One peak is apparent at 7.5 minutes (12%). One epimer of the DFTHU **1a** comes out at 9.5 minutes (350, 29%). The other epimer **1b** comes out at 14.3 minutes (370 mg, 31%). The deoxygenated product **26** elutes at 17 minutes (200 mg, 17%).

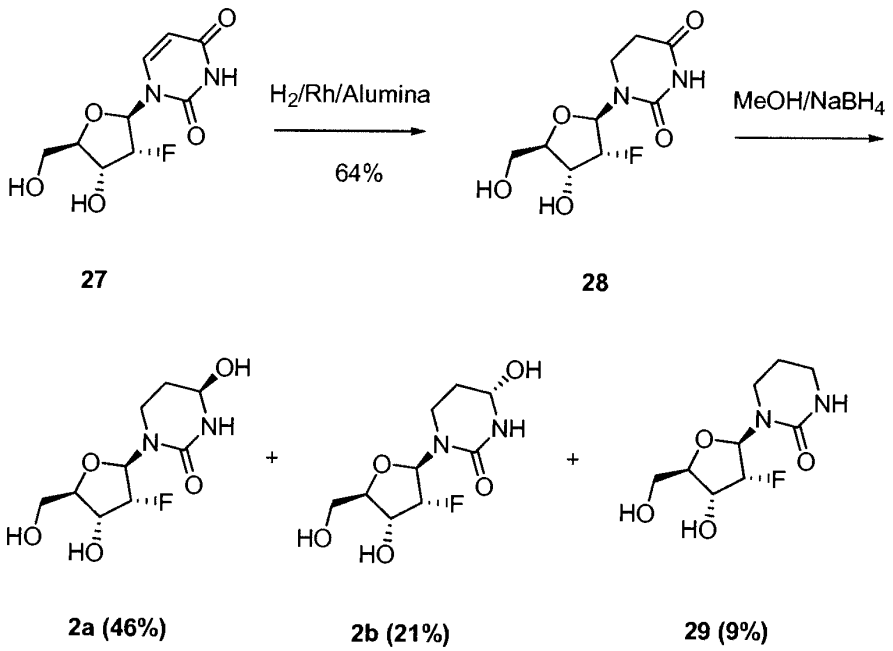
1a ^{1}HNMR (D_2O, 9.5 minutes): 6.03 (dd, 1H), 5.04 (bs, 1H), 4.20 (q, 1H), 3.90-3.71 (m, 3H), 3.53 (dt, 1H), 3.30 (dt, 1H), 1.92-1.75 (m, 2H). Anal. Calcd. for $C_9H_{14}N_2O_5F_2$ (0.15 H_2O): C, 39.90; H, 5.32; N, 10.34. Found: C, 39.87; H, 5.41; N, 10.26.

1b ^{1}HNMR (D_2O, 14.3 minutes): 5.97 (dd, 1H), 5.03 (bt, 1H), 4.16 (q, 1H), 3.91-3.68 (m, 3H), 3.41 (dt, 1H), 3.20 (dt, 1H), 1.95-1.80 (m, 2H). Anal Calcd. for $C_9H_{14}N_2O_5F_2$ (0.60 H_2O): C, 38.74; H, 5.49; N, 10.04. Found: C, 38.55; H, 5.36; N, 9.87.

26 ^{1}H NMR (D_2O) δ 5.99 (dd, J = 15 Hz, 6 Hz, 1H), 4.17 (m, 1H), 3.89 (m, 1H), 3.75 (m, 2H), 3.42 (m, 1H), 3.21 (t, J = 6 Hz, 2H), 3.18 (m, 1H), 1.86 (m, 2H).

Example 2:

Scheme 2. Synthesis of 2'(*R*)-fluoro-2'deoxy-tetrahydrouridines (Compounds 2a and 2b)



2'(*R*)-Fluoro-2'deoxy-DiHydroUridine [(*R*)-FDHU, 28]. 2'-Fluoro-2'-deoxyuridine **27** (1.2 g, 4.9 mmol) is dissolved in H_2O (30 mL) with a few drops of concentrated ammonium hydroxide (5 drops). Rhodium on alumina (300 mg) is added to the solution and the mixture is hydrogenated overnight at 40 psi. The next day, the mixture is filtered and the filtrate is concentrated and purified via prep HPLC (reverse phase C_{18} @ 5% CH_3CN/H_2O). The major product is **28, (*R*)-FDHU,** which elutes at 9.2 minutes (780 mg, 64%). Some residual starting material **7a** (5.5 minutes, 95 mg, 8%) and a minor amount of the FTHU **2a** and **2b** (7.2 minutes, 50 mg, 4% and 8.6 minutes, 45 mg, 4%) are isolated. ^{1}HNMR **28** (D_2O): 5.83 (dd, 1H), 5.07 (dd, 1H), 4.18 (q, 1H), 3.90-3.78 (m, 2H), 3.65 (dt, 1H), 3.52-3.35 (m, 2H), 2.64 (t, 2H).

2'(*R*)-Fluoro-2'-deoxy-TetraHydroUridine ((R)-FTHU, 2a and 2b). The (*R*)-FDHU (600 mg, 2.4 mmol) is dissolved in 20 mL of MeOH and cooled to 0 °C. Sodium borohydride (355 mg, 9.6 mmol) is added portion-wise to the solution and the reaction is slowly warmed to room temperature overnight. The MeOH is removed *in vacuo* and the residue is dissolved in 10 mL of H_2O. The solution is neutralized with 2.0 N HCl to pH 7. The solution is then purified via prep HPLC (reverse phase C_{18} @ 5% CH_3CN/H_2O). The desired product **2a** elutes at 7.2 minutes (275 mg, 46%) followed by the other epimer **2b** at 8.6 minutes (125 mg, 21%) and some residual starting material at 9.2 minutes and the fully reduced material **29** (50

mg, 9%) at 14.9 minutes. The stereochemistry at C-4 for 2a and 2b are assigned based on literature precedents on the crystal structure of cytidine deaminase in complex with a single epimer of tetrahydrouridine.

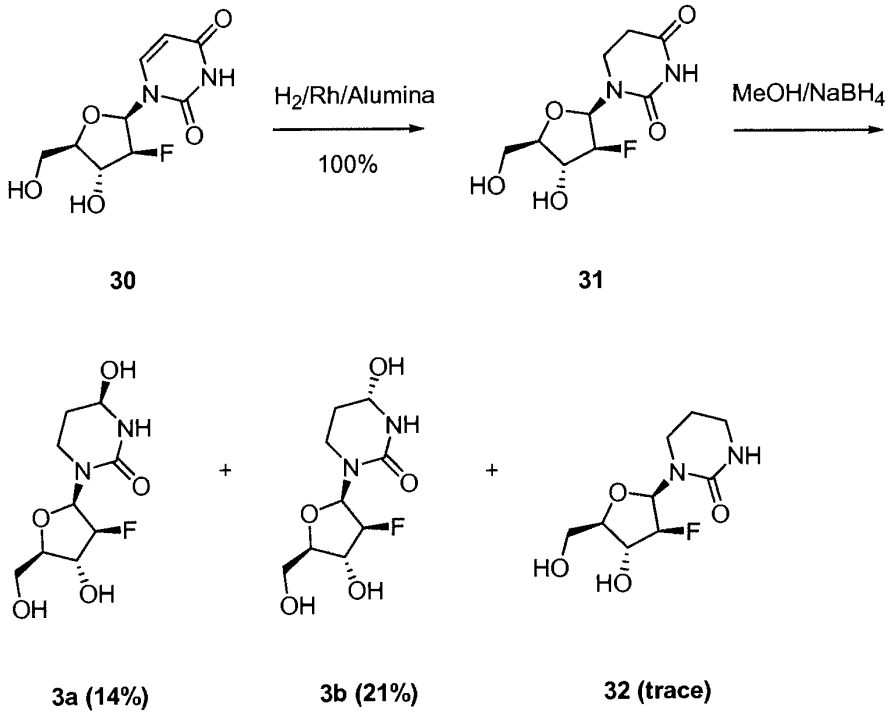
2a (7.2 minutes): ^{1}HNMR (DMSO-d_6): 7.21 (d, 1H), 5.93 (dd, 1H), 5.59 (d, 1H), 5.39 (d, 1H), 4.99-4.75 (m, 3H), 3.95 (m, 1H), 3.62-3.21 (m, 5H), 1.69 (m, 2H); ^{13}CNMR: 153.12, 91.2 (d), 85.93 (d), 81.36, 71.30, 68.3 (d), 60.43, 34.13, 28.66. Anal. Calcd. for $C_9H_{15}N_2O_5F$ (0.5 H_2O): C, 41.70; H, 6.22; N, 10.81. Found: C, 41.67; H, 6.26; N, 10.76.

2b (8.6 minutes): ^{1}HNMR (DMSO-d_6): 7.15 (d, 1H), 5.95 (dd, 1H), 5.58 (d, 1H), 5.40 (d, 1H), 5.00-4.75 (m, 3H), 3.92 (m, 2H), 3.61-3.29 (m, 5H), 2.98 (m, 1H), 1.80-1.65 (m, 2H); ^{13}CNMR: 154.02, 92.24 (d), 86.62 (d), 81.63, 71.73, 68.86 (d), 60.89, 35.08, 29.00.

29 (14.9 minutes): ^{1}HNMR (D_2O): 5.93 (dd, 1H), 5.07 (d, 1H), 4.61 (m, 1H), 4.24 (m, 1H), 3.96-3.65 (m, 3H), 3.35-3.14 (m, 3H), 2.12-1.79 (m, 2H).

Example 3:

Scheme 3. Synthesis of 2'(*S*)-fluoro-2'deoxy-tetrahydrouridines (Compounds 3a and 3b)



2'(*S*)-fluoro-2'deoxy-dihydrouridine [(*S*)-FDHU, 31]. Compound **30** (1.2g, 4.0 mmol) is dissolved in H_2O (40 mL). Rhodium on alumina (200 mg) is added to the solution and the mixture is hydrogenated overnight at 50 psi. The next day, the mixture is filtered through a pad of celite and concentrated *in vacuo*. The desired product **31** is obtained in quantitative

yield (>1.0 g). ^{1}HNMR (D_2O): 6.08 (dd, 1H), 5.09 (dt, 1H), 4.28 (m, 1H), 3.85-3.80 (m, 2H), 3.72 (m, 2H), 3.51 (m, 1H), 2.65 (t, J = 9 Hz, 2H).

2'(*S*)-fluoro-2'deoxy-tetrahydrouridine [(*S*)-FTHU, 3a and 3b]. Compound **31** (1.12 mg, 4.55 mmol) is dissolved in 28 mL of MeOH and cooled to 0°C. Sodium borohydride (475 mg, 12.55 mmol) is added portion-wise to the solution and the reaction is allowed to continue for 1 hour and 15 minutes. The MeOH is removed *in vacuo* and the residue is dissolved in 15 mL of 5% CH_3CN/H_2O. The solution is neutralized with 2.0 N HCl to pH 7 (~3 ml). The solution is then purified via prep HPLC (reverse phase C_{18} Phenomenex Luna with a 5% CH_3CN/H_2O (isocratic eluent and refractive index detector). The desired product **3a** elutes at 9.3 minutes (163 mg, 14%) followed by the other epimer **3b** at 13.4 minutes (236 mg, 21%), some residual starting material (not quantified), as well as the fully reduced product **32** (not quantified) are detected. Stereochemistry at C-4 for 3a and 3b are assigned based on literature precedents on the crystal structure of cytidine deaminase in complex with a single epimer of tetrahydrouridine.

3a (9.3 minutes): ^{1}HNMR (D_2O) δ 6.12 (dd, 1H), 5.04 (dt, 1H), 5.03 (m, 1H), 4.27 (m, 1H), 3.83-3.59 (m, 4H), 3.34 (m, 1H), 1.86 (m, 2H). ^{13}CNMR: 157.9, 98.6 (d), 83.9 (d), 82.5, 75.7 (d), 74.0, 62.6, 37.5, 30.0. Anal. Calcd. for $C_9H_{15}N_2O_5F$ (0.25 H_2O): C, 42.44; H, 6.13; N, 11.00. Found: C, 42.49; H, 6.09; N, 10.82.

3b (13.4 minutes): ^{1}HNMR (D_2O) δ 6.07 (dd, 1H), 5.02 (dt, 1H), 5.02 (t, 1H), 4.26 (dt, 1H), 3.84-3.66 (m, 4H), 3.35 (m, 1H), 1.86 (m, 2H); ^{13}CNMR: 157.8, 98.2 (d), 84.4, 82.0, 75.7, 74.0, 62.4, 38.4, 29.3. Anal. Calcd. for $C_9H_{15}N_2O_5F$ (0.4 H_2O): C, 41.96; H, 6.19; N, 10.87. Found: C, 41.99; H, 6.15; N, 10.91.

Example 4:

CDA enzymatic activity

The ability of the compounds of the invention to inhibit the enzymatic activity of CDA may be demonstrated using the following assay method.

The procedure to determine CDA enzymatic activity is based on published methodologies (for example, Cacciamani, T. *et al.*, *Arch. Biochem. Biophys.* **1991**, *290*, 285-92; Cohen R. *et al.*, *J. Biol. Chem.*, **1971**, *246*, 7566-8; Vincenzetti S. *et al.*, *Protein Expr. Purif.* **1996**, *8*, 247-53). The assay follows the change in absorbance at 286 nm of the CDA-catalyzed deamination of cytidine to form uridine. The reaction is carried out in potassium phosphate buffer (pH 7.4, 20 mM, containing 1 mM DTT) in a total volume of 200 µl in a 96-well plate format. The final reaction mixture contains cytidine (50 µM) and purified human recombinant CDA. Purified enzyme is diluted so as to produce an absorbance change of

approximately 2 milli-absorbance units / minute. Base line measurements of absorbance change over time are made before CDA addition to insure no change of absorbance in the absence of CDA. After CDA addition, absorbance change is monitored for 20 – 30 minutes. When potential inhibitors are present, eight concentrations of each in the 0.1 nM – 1 mM range are tested in order to obtain IC_{50} values. The slope of the change of absorbance over time for samples containing both cytidine and CDA but no inhibitor (totals) is normalized to 100%. CDA enzymatic activity left in the presence of a compound expressed as percent of total activity is subtracted from 100% in order to obtain percent inhibition at varying compound concentrations.

Using the above described assay, the inhibitory potency of Compounds **1** and **2** are evaluated. The IC_{50} values of the compounds are set forth in Table 1. "1a" and "1b" denote single stereoisomers; "1" denotes an epimeric mixture.

Table 1. Inhibitory Potency of Test Compounds

Compound	IC_{50} (nM)
1a	400 ± 60
1b	5000 ± 1000
1	400 ± 60
2a	200 ± 50
2b	5000 ± 2000
2	2000 ± 3000
3a	400 ± 80
3b	4000 ± 700
3	2000 ± 1000

Enhancement of Efficacy of CDA Substrate Drugs

The ability of the compounds of the invention to enhance the efficacy of CDA substrate drugs may be demonstrated in the L1210 mouse lymphoma model.

Example 5:

The effect of CDA inhibitor, Compound 1, on decitabine (0.1mg/kg) induced survival in the L1210 survival model

Methods

30 CD2F1 6-7 weeks old female mice are randomly separated into 6 groups:

Group #	
1	L1210 i.v and Vehicle + Vehicle p.o. x 2 for 4 days
2	L1210 i.v. and Vehicle + Compound **1** 10 mg/kg p.o. x 2 for 4 days
3	L1210 i.v. and Vehicle + 0.1 mg/kg decitabine p.o. x 2 for 4 days
4	L1210 i.v. and Compound **1** 1 mg/kg + 0.1 mg/kg decitabine p.o. x 2 for 4 days
5	L1210 i.v. and Compound **1** 10mg/kg + 0.1 mg/kg decitabine p.o. x 2 for 4 days
6	L1210 i.v. and Vehicle + 0.1 mg/kg decitabine i.p. x 2 for 4 days

L1210 Intravenous (i.v.) Injection: Prior to experiment, L1210 cells are passed at least 3 times in CD2F1 female mice. The mice are injected intraperitoneally (i.p.) with L1210 ascites one prior week to sacrifice using CO_2. Each mouse is placed on its back, its belly surface is cleaned with alcohol wipes and a small incision is made into its peritoneal cavity. 2 ml of ice cold 2.1% bovine serum albumin (BSA) in saline is injected into the cavity. Fluid is withdrawn from the cavity, transferred with an 18G 3 cc syringe into a clean sterile tube, and kept on ice. The fluid is diluted 1:10 in 2.1% BSA in saline and one drop of zap-o-globin is added to 2 ml of diluted ascites. Diluted ascites (dilute 1:10 again) are counted on a hematocytomer and the number of cells/ml is calculated. A stock of ascites in BSA solution is diluted to $1x10^4$ cells/0.1 ml. Mice are injected with 0.1 ml of cell solution with a 27G needle.

Dose Solution Preparation: When appropriate, mice are dosed with a vehicle or Compound **1** p.o 30 minutes prior to decitabine.

Compound **1** is prepared at 1 mg/ml in phosphate buffer saline (PBS) and then diluted to 0.1 mg/ml in PBS for the lower dose.

Decitabine is prepared at a 1 mg/ml stock in PBS and diluted appropriately to achieve a 0.01 and 0.02 mg/ml dosing solution.

Dosing Scheme: Decitabine is prepared fresh twice a day. All dose solutions are stored on ice while dosing. Mice are dosed i.p. or orally (p.o.) twice a day (8 hours apart) for 4 consecutive days. Final dosing scheme and total decitabine and Compound **1** doses are outlined in Table 2.

Table 2. Dosing Scheme

Group #	Drug	Decitabine Dose (route administered)	Cumulative Decitabine Dose	Compound 1 Dose	Cumulative Compound 1 Dose
1	Vehicle	Vehicle	0 mg/kg	Vehicle	0 mg/kg
2	Compound **1**	Vehicle	0 mg/kg	10 mg/kg	80 mg/kg
3	Decitabine	0.1 mg/kg p.o.	0.8 mg/kg	Vehicle	0 mg/kg
4	Decitabine / Compound **1**	0.1 mg/kg p.o.	0.8 mg/kg	1 mg/kg	40 mg/kg
5	Decitabine / Compound **1**	0.1 mg/kg p.o.	0.8 mg/kg	10 mg/kg	80 mg/kg
6	Decitabine	0.1 mg/kg i.p.	0.8 mg/kg	Vehicle	0 mg/kg

Survival and Autopsy: Mice are observed for survival and weighed daily (Monday to Friday) for the duration of the study (30 days). Dead mice are autopsied and observed for the presence of tumors in organs. Tumor deaths are determined by liver weights greater than 1.6 g and spleen weights greater than 150 mg as per Covey *et al.*, *Eur. J. Cancer Oncol.* 1985.

Mice dosed with decitabine or decitabine plus Compound **1** live longer than mice dosed with vehicle control or Compound **1** alone (FIG. 1 and Table 3; $p<0.05$). A trend for a dose response is observed with Compound **1** in combination with decitabine.

Decitabine (0.1 mg/kg) p.o. is less effective than 0.1 mg/kg decitabine i.p., but not significantly different (Table 3; FIG. 1, $p=0.052$).

Co-administration of 10 mg/kg Compound **1** with 0.1 mg/kg decitabine p.o. significantly enhances survival compared to 0.1 mg/kg decitabine p.o. alone ($p=0.0031$) and 0.1 mg/kg decitabine i.p. ($p=0.016$; Table 3, FIG. 1). Co-administration of 1 mg/kg Compound **1** with 0.1 mg/kg decitabine p.o. significantly enhances survival compared to 0.1

mg/kg decitabine p.o. alone (p=0.0031), but not compared to 0.1 mg/kg decitabine i.p. (p=0.13; Table 3, FIG. 1).

Table 3 lists the mean survival of each treatment group and the percent ILS (increased life span) compared to the vehicle group. All treated groups live significantly longer than vehicle controls and CDA inhibitor alone groups ($p<0.05$).

Table 3 lists the weights of the livers and spleens of mice on autopsy. All mice except for the one 0.1 mg/kg decitabine i.p. mouse died a 'tumor burden' related death as indicated by the liver weights greater than 1 g and the spleen weights greater than 80 mg (Covey *et al.*, *supra*). The weights of liver and spleen from 3 control mice are 0.97±0.08g and 0.08±0.02g. Gross observations are noted concerning the overall appearance of the peritoneal and thoracic cavities.

Table 3. Effect of Decitabine and Compound 1 on Survival and Liver and Spleen Weights in L1210 IV Survival Model

Group #	L1210 cells	Mean Survival (days) ± SD	* % ILS (Increased Life Span)	Mean Liver wts. (g) ± SD	Mean Spleen wts. (g) ± SD
1	$1x10^4$	7.40±0.55	n/a	1.81±0.13	0.31±0.05
2	$1x10^4$	7.40±0.55	0.00	1.99±0.22	0.39±0.07
3	$1x10^4$	10.6±0.56	43.24	2.05±0.17	0.33±0.06
4	$1x10^4$	12.8±0.45	72.97	2.03±0.08	0.29±0.03
5	$1x10^4$	14.2±0.45	91.89	2.02±0.27	0.29±0.07

* % ILS = $\frac{\text{mean survival of experimental (days)} - \text{mean survival of control (days)} \times 100}{\text{mean survival of control (days)}}$

FIG. 1 is a graph that shows the effect of Compound 1 on decitabine (DAC) induced survival in the L1210 mouse lymphoma model.

Example 6:

The effect of CDA inhibitor, Compound 1a, on decitabine (0.1mg/kg) induced survival in the L1210 survival model

Compound 1a is evaluated in the L1210 model following the protocol of Example 5. Mice dosed with decitabine ("DAC"), and DAC plus Compound 1a, live longer than those receiving

vehicle control and CDA inhibitor alone (FIG. 2; $p < 0.05$). In combination with DAC, 10 mg/kg Compound 1a is more effective at extending survival than the lower doses.

Example 7:

The effect of CDA inhibitor, Compound 3a, on decitabine (0.1mg/kg) induced survival in the L1210 survival model

Compound 3a is evaluated in the L1210 model following the protocol of Example 5. Mice dosed with decitabine ("DAC"), and DAC plus Compound 3a live longer than vehicle controls and CDA inhibitor alone (FIG. 3; $p < 0.05$).

Example 8:

The effect of CDA inhibitor, Compound 1 on cytarabine (ara-C) induced survival in the L1210 survival model

50 CD2F1 6-7 weeks old female mice are randomly separated into 10 groups. (N = 5 mice/group):

Group #	
1	L1210 i.v. and Veh + Veh (PBS) p.o. x 2 for 4 days
2	L1210 i.v. and Veh + Ara-C 200 mg/kg p.o. x 2 for 4 days
3	L1210 i.v. and Veh + Ara-C 100 mg/kg p.o. x 2 for 4 days
4	L1210 i.v. and Veh + Ara-C 50 mg/kg p.o. x 2 for 4 days
5	L1210 i.v. and Veh + Ara-C 25 mg/kg p.o x 2 for 4 days
6	L1210 i.v. and Compound 1 10 mg/kg + Ara-C 200mg/kg x 2 for 4 days
7	L1210 i.v. and Compound 1 10 mg/kg + Ara-C 100mg/kg x 2 for 4 days
8	L1210 i.v. and Compound 1 10 mg/kg + Ara-C 50 mg/kg x 2 for 4 days
9	L1210 i.v. and Compound 1 10 mg/kg + Ara-C 25mg/kg x 2 for 4 days

Group #	
10	L1210 i.v. and Compound 1 10 mg/kg + Veh p.o. x 2 for 4 days

L1210 IV injection: CD2F1 female mice are injected i.p. with L1210 ascites one prior week to sacrifice (CO_2). L1210 cells are passed at least 3 times *in vivo* prior to experiment. The mouse is placed on its back, belly surface cleaned with alcohol wipes and a small incision made into peritoneal cavity. 2 ml of ice cold 2.1% BSA in saline is injected into cavity and then fluid withdrawn and transferred with an 18G 3cc syringe into a clean sterile tube and kept on ice. The fluid is diluted 1:10 in 2.1% BSA in saline and one drop of zap-o-globin is added to 2 ml of diluted ascites. Diluted ascites (dilute 1:10 again) are counted on a hematocytomer and the number of cells/ml is calculated. Stock of ascites in BSA solution is diluted to $1x10^4$ cells/0.1 ml. Mice are injected with 0.1 ml of cell solution with 27G needle. Total i.v. injections take about 50 minutes.

Dose Solution Preparation: When appropriate mice are dosed with vehicle or Compound 1 p.o. 30 minutes prior to Ara-C. Compound 1 is prepared at 1 mg/ml in PBS and Ara-C is prepared at a 20 mg/ml stock in PBS and then diluted appropriately for lower doses.

Dosing Scheme: Compound 1 is prepared at the beginning of the study and stored at 4°C. Ara-C is prepared fresh twice a day. All solutions are stored on ice while dosing. Mice are dosed orally twice a day (8 hours apart) for 4 consecutive days. Final dosing scheme and total Ara-C and Compound 1 dose is outlined in Table 4.

Table 4. Dosing Scheme

Group #	Drug	Ara-C Dose (route adm.)	Cumulative Ara-C Dose	Compound 1 Dose	Cumulative Compound 1 Dose
1	Veh	Veh	0 mg/kg	Veh	0 mg/kg
2	Compound 1	Veh	0 mg/kg	10 mg/kg	40 mg/kg
3	Ara-C	200 mg/kg p.o.	800 mg/kg	Veh	0 mg/kg
4	Ara-C	100 mg/kg p.o.	400 mg/kg	Veh	0 mg/kg
5	Ara-C	50 mg/kg p.o.	200 mg/kg	Veh	0 mg/kg
6	Ara-C	25 mg/kg p.o.	100 mg/kg	Veh	0 mg/kg
7	Ara-C	200 mg/kg p.o.	800 mg/kg	10 mg/kg	40 mg/kg
8	Ara-C	100mg/kg p.o.	400 mg/kg	10 mg/kg	40 mg/kg
9	Ara-C	50 mg/kg p.o.	200 mg/kg	10 mg/kg	40 mg/kg
10	Ara-C	25 mg/kg p.o.	100 mg/kg	10 mg/kg	40 mg/kg

Survival and Autopsy: Mice are observed for survival and weighed daily (Mon-Fri) for the duration of the study (45 days). Dead mice are autopsied and observed for the presence of tumors in organs. Tumor deaths are determined by liver weights greater than 1.6 g and spleen weights greater than 150 mg as per Covey *et al.*, *supra*.

Mice dosed with Ara-C alone (50 mg/kg, 100 mg/kg and 200 mg/kg) and Ara-C plus Compound 1 live longer than mice treated with vehicle control and CDA inhibitor alone (FIG.'s 4-7; $p<0.05$). Compound 1 alone has no effect on survival compared to vehicle controls (FIG. 4).

25 mg/kg Ara-C alone has no effect at extending survival compared to mice treated with vehicle and 10 mg/kg Compound 1 . 50, 100 and 200 mg/kg Ara-C significantly enhance survival (days) in a dose dependent manner compared to mice in the control group. Co-administration of 10 mg/kg Compound 1 with Ara-C p.o. significantly enhances survival compared to the survival time of mice treated with the same dose of Ara-C alone (FIG.'s 4-7).

Example 9:

Effect of Compound 1 on gemcitabine-induced reduction of tumor volume in the mouse A2780 human ovarian cancer xenograft model

The oral efficacy of gemcitabine is tested in combination with Compound 1 in the human ovarian cancer xenograft A2780. Female NCr nu/nu 5-6 week old mice are implanted subcutaneously with 30 to 60 mg tumor fragments. On day 11 when the tumors are approximately 200 mm^3, treatment starts as described in Table 5.

Table 5. Dosing Scheme

Groups	Treatment	Gemcitabine Schedule	Compound 1 Schedule
1	Vehicle (saline)	PO; q3dx4	
2	Compound 1		PO 10 mg/kg q3dx4
3	Gemcitabine	PO 10 mg/kg; q3dx4	
4	Gemcitabine/Compound 1*	PO 10 mg/kg; q3dx4	PO 10 mg/kg q3dx4
5	Gemcitabine	PO 30 mg/kg; q3dx4	
6	Gemcitabine/Compound 1*	PO 30 mg/kg; q3dx4	PO 10 mg/kg q3dx4

- Compound 1 is dosed approximately 30 min prior to Gemcitabine

Tumor volume is followed throughout the experiment. Tumor volume is measured three times weekly. Tumor burden (mg = mm^3) is calculated from caliper measurements by

the formula for the volume of a prolate ellipsoid $(LxW^2)/2$ where L and W are the respective orthogonal length and width measurements (mm).

The primary endpoints used to evaluate efficacy in the A2780 model are complete and partial tumor regressions, tumor growth delay and the number of tumor free survivors at the end of the study. A complete response (CR) is defined as a decrease in tumor size to an undetectable size (< $50mm^3$). A partial response (PR) is defined as >50% decrease in tumor mass form starting tumor size. A tumor that achieves a CR or PR during the study but starts to grow again is still considered a CR or PR. Tumor free survival (TFS) at the end of the study would be no detectable tumor (<50 mm^3) at study termination (day 74). Tumor growth delay (TGD) is defined in this experiment as the median number of days for the treatment group compared to the control group to reach 750 mm^3.

Oral administration of 10 mg/kg PO q3dx4 gemcitabine is not very effective in this model with a tumor growth delay of 6.1 days (not statistically significant), with no CRs, PRs or tumor free survivors at termination of the experiment at day 74. When 10 mg/kg Gemcitabine is dosed in combination with Compound 1, significant delay of tumor growth is observed compared to this dose of gemcitabine alone (19.2 days; $p<0.05$ compared to Gem alone) with 75% of tumors displaying CR, but there are no TFS at the end of the experiment. In FIG. 8 (Tumor Volume vs Time (days) post treatment start, it is apparent that Compound 1 alone has no effect on tumor growth, while combination treatment with Compound 1 and gemcitabine is more effective than gemcitabine alone.

Oral administration of 30 mg/kg PO q3dx4 (MTD) gemcitabine is effective in producing a statistically significant tumor growth delay of 22 days with 63% CRs but no TFS. Combination chemotherapy with gemcitabine (30 mg/kg PO) plus Compound 1 produced a statistically significant tumor growth delay of > 57 days and 100% CR and a 50% incidence of TFS at day 74, termination of the experiment.

Table 6. Effect of Combination treatment Gemcitabine and Compound 1 on Tumor Growth delay in the A2780 Ovarian cancer model

Treatment	TGD	P value
Untreated Control	NA	
10 mg/kg Compound 1	0.4 days	
10 mg/kg Gemcitabine	6.1 days	
10 mg/kg Gemcitabine	19.2 days	<0.05 compared to 10 mg/kg Gem alone

Treatment	TGD	P value
+ Compound 1		
30 mg/kg Gemcitabine	22 days	<0.05 compared to Untreated control
30 mg/kg Gemcitabine + Compound 1	> 57 days	<0.05 compared to 30 mg/kg Gem alone

Example 10:

Solid state characterization of Compound 1a

Data Collection

A colorless prism crystal of Compound 1a ($F_2O_5N_2C_9H_{14}$) having approximate dimensions of 0.48 x 0.43 x 0.32 mm is mounted in a loop. All measurements are made on a Rigaku RAXIS SPIDER imaging plate area detector with graphite monochromated Cu-Kα radiation.

Indexing is performed from 5 oscillations that are exposed for 60 seconds. The crystal-to-detector distance is 127.40 mm.

Cell constants and an orientation matrix for data collection correspond to a primitive trigonal cell (laue class: -3m1) with dimensions:

$$a = 9.78961(18)\ \text{Å}$$
$$c = 20.4588(7)\ \text{Å}$$
$$V = 1698.02(7)\ \text{Å}^3$$

For Z = 6 and F.W. = 268.22, the calculated density is 1.574 g/cm^3. Based on the systematic absences of:

$$000l: l \pm 3n$$

and the successful solution and refinement of the structure, the space group is determined to be:

$$P3_121\ (\#152)$$

The data is collected at a temperature of -123 ± 1°C to a maximum 2θ value of 136.4°. A total of 111 oscillation images are collected. A sweep of data is done using ω scans from 20.0 to 200.0° in 5.0° steps, at χ=0.0° and φ = 180.0°. A second sweep is performed from

20.0 to 200.0° in 5.0° steps, at χ=54.0° and ϕ = 180.0°. A third sweep is performed from 20.0 to 185.0° in 5.0° steps, at χ=54.0° and ϕ = 90.0°, and a final sweep is performed using ω scans from 20.0 to 50.0° in 5.0° steps, at χ=0.0° and ϕ = 0.0°. The exposure rate is 12.0 [sec./°]. The crystal-to-detector distance is 127.40 mm. Readout is performed in the 0.100 mm pixel mode.

Data Reduction

Of the 11772 reflections that are collected, 2052 are unique (R_{int} = 0.038); equivalent reflections are merged.

The linear absorption coefficient, μ, for Cu-Kα radiation is 13.035 cm^{-1}. An empirical absorption correction is applied which results in transmission factors ranging from 0.540 to 0.659. The data is corrected for Lorentz and polarization effects. A correction for secondary extinction (Larson, A.C., *Crystallographic Computing* **1970,** 291-294; equation 22, with V replaced by the cell volume) is applied (coefficient = 0.005900).

Structure Solution and Refinement

The structure is solved by direct methods (SIR92: Larson, A.C., *J. Appl. Cryst.*, **1994,** *27*, 435) and expanded using Fourier techniques(DIRDIF99: Beurskens, P.T. *et al., The DIRD-99 Program System. Technical Report of the Crystallography Laboratory*, **1999,** University of Nijmegen, The Netherlands). The non-hydrogen atoms are refined anisotropically. Some hydrogen atoms are refined isotropically and the rest are refined using the riding model. The final cycle of full-matrix least-squares refinement (least squares weights = $\Sigma w(F_o^2-F_c^2)^2$) on F^2 is based on 2052 observed reflections and 181 variable parameters and converges (largest parameter shift is <0.01 times its esd) with unweighted and weighted agreement factors of:

$$R1 = \Sigma \, ||Fo| - |Fc|| \, / \, \Sigma \, |Fo| = 0.0303$$

$$wR2 = [\, \Sigma \, (\, w \, (Fo^2 - Fc^2)^2 \,) / \, \Sigma \, w(Fo^2)^2]^{1/2} = 0.0733$$

The standard deviation of an observation of unit weight (standard deviation = $[\Sigma w(F_o^2-F_c^2)^2/(N_o-N_v)]^{1/2}$, N_o = number of observations, N_v = number of variables) is 1.10. Unit weights are used. The maximum and minimum peaks on the final difference Fourier map correspond to 0.22 and -0.22 $e^-/Å^3$, respectively. The absolute structure is deduced based on Flack parameter, 0.0(1), refined using 839 Friedel pairs (Flack, H. D., *Acta Cryst.* **1983,** *A39*, 876-881.

Neutral atom scattering factors are taken from Cromer, D. T. *et al.*, *International Tables for X-ray Crystallography* **1974,** *IV*, Table 2.2 A. Anomalous dispersion effects are included in Fcalc (Ibers, J. A. *et al., Acta Crystallogr.* **1964**, *17*, 781); the values for Δf' and Δf" are those of Creagh, D. C. *et al..*, *International Tables for Crystallography* **1992,** *C*, Table 4.2.6.8, 219-222. The values for the mass attenuation coefficients are those of Creagh, D. C. *et al.. International Tables for Crystallography* **1992**, *C*, Table 4.2.4.3, 200-206. All calculations are performed using the CrystalStructure 3.8 crystallographic software package except for refinement, which is performed using SHELXL-97.

Experimental Details

A. Crystal Data

Empirical Formula	$F_2O_5N_2C_9H_{14}$
Formula Weight	268.22
Crystal Color, Habit	colorless, prism
Crystal Dimensions	0.48 X 0.43 X 0.32 mm
Crystal System	trigonal
Lattice Type	Primitive
Indexing Images	5 oscillations @ 60.0 seconds
Detector Position	127.40 mm
Pixel Size	0.100 mm
Lattice Parameters	a = 9.78961(18) Å c = 20.4588(7) Å V = 1698.02(7) Å^3
Space Group	$P3_121$ (#152)
Z value	6
D_{calc}	1.574 g/cm^3
F_{000}	840.00
μ(CuKα)	13.035 cm^{-1}

B. Intensity Measurements

Diffractometer	Rigaku RAXIS-SPIDER
Radiation	CuKα (λ = 1.54187 Å) graphite monochromated
Detector Aperture	460 mm x 256 mm

Data Images	111 exposures
ω Oscillation Range (χ=0.0, ϕ=180.0)	20.0 - 200.0°
ω Oscillation Range (χ=54.0, ϕ=180.0)	20.0 - 200.0°
ω Oscillation Range (χ=54.0, ϕ=90.0)	20.0 - 185.0°
ω Oscillation Range (χ=0.0, ϕ=0.0)	20.0 - 50.0°
Exposure Rate	12.0 sec./°
Detector Position	127.40 mm
Pixel Size	0.100 mm
$2\theta_{Max}$	136.4°
No. of Reflections Measured	Total: 11772 Unique: 2052 (R_{int} = 0.038) Friedel pairs: 839
Corrections	Lorentz-polarization Absorption (trans. factors: 0.540 - 0.659) Secondary Extinction (coefficient: 5.90000e-003)

C. Structure Solution and Refinement

Structure Solution	Direct Methods (SIR92)
Refinement	Full-matrix least-squares on F^2
Function Minimized	Σ w $(Fo^2 - Fc^2)^2$
Least Squares Weights	$w = 1/ [\sigma^2(Fo^2) + (0.0317 \cdot P)^2 + 0.6904 \cdot P]$ where $P = (Max(Fo^2,0) + 2Fc^2)/3$
$2\theta_{Max}$ cutoff	136.4°
Anomalous Dispersion	All non-hydrogen atoms
No. Observations (All Reflections)	2052
No. Variables	181
Reflection/Parameter Ratio	11.34
Residuals: R1 (I>2.00σ(I))	0.0303
Residuals: R (All Reflections)	0.0329

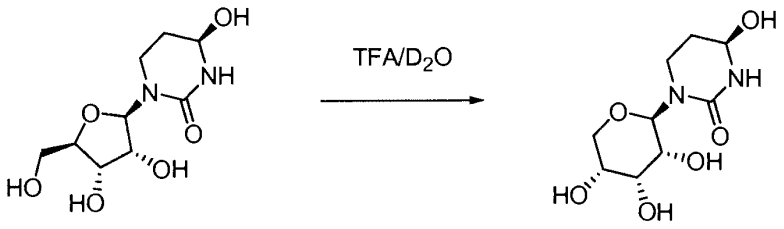
Residuals: wR2 (All Reflections)	0.0733
Goodness of Fit Indicator	1.099
Flack Parameter (Friedel pairs = 839)	0.0(1)
Max Shift/Error in Final Cycle	<0.001
Maximum peak in Final Diff. Map	0.22 $e^-/Å^3$
Minimum peak in Final Diff. Map	-0.22 $e^-/Å^3$

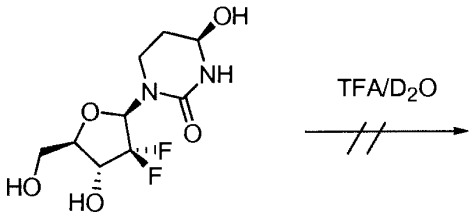
An ORTEP plot (Michael N. Burnett and Carroll K. Johnson, ORTEP-III: Oak Ridge Thermal Ellipsoid Plot Program for Crystal Structure Illustrations, Oak Ridge National Laboratory Report ORNL-6895, 1996) of the determined structure of Compound 1a is shown in FIG. 9.

Example 11:

Enhancement of acid stability of Compound 1a compared to THU

The stability of Compound 1a and tetrahydrouridine (THU) in acid solution are evaluated by ^{1}H NMR spectroscopy.





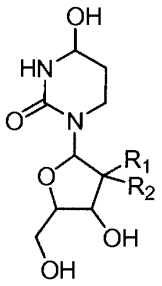
Tetrahydrouridine (THU, 5 mg) is dissolved in D_2O (0.75 mL). The ^{1}HNMR (D_2O, 300 MHz, 27° C) spectrum is shown in FIG. 10. To this same sample is added one drop of deuterated TFA followed by an immediate ^{1}HNMR spectrum (FIG. 10). Even at this earliest time point, the peak at 5.4 ppm (~10% conversion by integration) is indicative of the ring expansion to the **TH-pyranose.** Over the next several hours, spectra (D_2O, 300 MHz, 27° C) are taken as shown in FIG. 11. After 4 hours, the **TH-pyranose** is more prevalent indicating about 60% conversion. At 72 hours, the conversion is almost entirely complete (>80%).

Notable changes in the region from 4.0-4.5 are also indicative of **THU** decomposition and the **TH-pyranose** formation.

Compound 1a (5 mg) is dissolved in D_2O (0.75 mL). The ^{1}HNMR spectrum (D_2O, 300 MHz, 27° C) is shown in FIG. 12. To this same sample is added one drop of deuterated TFA followed by an immediate ^{1}HNMR spectrum (FIG. 12). After the first time point, the only notable change is the epimerization of the aminal which is noted by the extra peaks at 5.92 and 5.93. After 4 hours and 72 hours (FIG. 13), there are no other notable changes in the spectra (D_2O, 300 MHz, 27° C). These results indicate that no pyranose formation occurs with Compound 1a under these conditions.

Certain Embodiments of the Invention

1. A compound of formula I



I

or a pharmaceutically acceptable salt of the compound, wherein:

R_1 and R_2 are independently selected from the group consisting of hydrogen, halo, cyano, nitro, sulfhydryl, hydroxyl, formyl, carboxyl, COO(C_1 to C_6 straight or branched chain alkyl), COO(C_1 to C_6 straight or branched chain alkenyl), COO(C_1 to C_6 straight or branched chain alkynyl), CO(C_1 to C_6 straight or branched chain alkyl), CO(C_1 to C_6 straight or branched chain alkenyl), CO(C_1 to C_6 straight or branched chain alkynyl), C_1 to C_6 straight or branched chain alkyl, C_1 to C_6 straight or branched chain alkenyl, C_1 to C_6 straight or branched chain alkynyl, C_1 to C_6 straight or branched chain alkoxy, and C_1 to C_6 straight or branched chain alkenoxy; wherein each occurrence of C_1 to C_6 straight or branched chain alkyl, C_1 to C_6 straight or branched chain alkenyl, C_1 to C_6 straight or branched chain alkynyl, C_1 to C_6 straight or branched chain alkoxy, or C_1 to C_6 straight or branched chain alkenoxy may be independently unsubstituted or substituted with one to four substituents independently selected from the group consisting of halo, hydroxyl, cyano, nitro, formyl, carboxyl, and sulfhydryl;

and provided that when one of R_1 and R_2 is -H, then the other is not -H, -OH or $-CH_2OH$.

2. A compound of embodiment 1, wherein R_1 and R_2 are independently selected from the group consisting of hydrogen, halo, hydroxyl, cyano, nitro, sulfhydryl, C_1 to C_6 straight or branched chain alkyl, C_1 to C_6 straight or branched chain alkenyl, C_1 to C_6 straight or branched chain alkynyl, C_1 to C_6 straight or branched chain alkoxy, and C_1 to C_6 straight or branched chain alkenoxy; wherein wherein each occurrence of C_1 to C_6 straight or branched chain alkyl, C_1 to C_6 straight or branched chain alkenyl, C_1 to C_6 straight or branched chain alkynyl, C_1 to C_6 straight or branched chain alkoxy, or C_1 to C_6 straight or branched chain alkenoxy may be independently unsubstituted or substituted with one ore more halos;

and provided that when one of R_1 and R_2 is -H, then the other is not -H, or -OH.

3. A compound of embodiment 1, wherein R_1 and R_2 are independently selected from the group consisting of hydrogen, halo, C_1 to C_6 alkyl, C_1 to C_6 alkenyl, C_1 to C_6 alkoxy, and C_1 to C_6 alkenoxy; wherein each occurrence of C_1 to C_6 straight or branched chain alkyl, C_1 to C_6 straight or branched chain alkenyl, C_1 to C_6 straight or branched chain alkoxy and C_1 to C_6 straight or branched chain alkenoxy may be independently unsubstituted or substituted with one to three halos;

and provided that when one of R_1 and R_2 is -H, then the other is not -H, or -OH.

4. The compound of embodiment 1, wherein at least one of R_1 and R_2 is halo.

5. The compound of embodiment 1, wherein at least one of R_1 and R_2 is fluoro.

6. The compound of embodiment 1, wherein one of R_1 and R_2 is halo, and the other is -H.

7. The compound of embodiment 1, wherein one of R_1 and R_2 is fluoro, and the other is -H.

8. The compound of embodiment 1, wherein R_1 and R_2 are each fluoro.

9. The compound of embodiment 1, wherein one of R_1 and R_2 is halo, and the other is -CN.

10. The compound of embodiment 1, wherein one of R_1 and R_2 is fluoro, and the other is -CN.

11. The compound of embodiment 1, wherein one of R_1 and R_2 is halo, and the other is $-NO_2$.

12. The compound of embodiment 1, wherein one of R_1 and R_2 is fluoro, and the other is $-NO_2$.

13. The compound of embodiment 1, wherein one of R_1 and R_2 is halo, and the other is -SH.

14. The compound of embodiment 1, wherein one of R_1 and R_2 is fluoro, and the other is -SH.

15. The compound of embodiment 1, wherein one of R_1 and R_2 is halo, and the other is -OH.

16. The compound of embodiment 1, wherein one of R_1 and R_2 is fluoro, and the other is -OH.

17. The compound of embodiment 1, wherein one of R_1 and R_2 is halo, and the other is -CHO.

18. The compound of embodiment 1, wherein one of R_1 and R_2 is fluoro, and the other is -CHO.

19. The compound of embodiment 1, wherein one of R_1 and R_2 is halo, and the other is -COOH.

20. The compound of embodiment 1, wherein one of R_1 and R_2 is fluoro, and the other is -COOH.

21. The compound of embodiment 1, wherein one of R_1 and R_2 is halo, and the other is $-COOR_x$, and wherein R_x selected from the group consisting of C_1 to C_6 straight or branched chain alkyl, C_1 to C_6 straight or branched chain alkenyl, and C_1 to C_6 straight or branched chain alkynyl.

22. The compound of embodiment 1, wherein one of R_1 and R_2 is fluoro, and the other is $-COOR_x$ and wherein R_x selected from the group consisting of C_1 to C_6 straight or branched chain alkyl, C_1 to C_6 straight or branched chain alkenyl, and C_1 to C_6 straight or branched chain alkynyl.

23. The compound of embodiment 1, wherein one of R_1 and R_2 is halo, and the other is $-COR_x$, and wherein R_x selected from the group consisting of C_1 to C_6 straight or branched chain alkyl, C_1 to C_6 straight or branched chain alkenyl, and C_1 to C_6 straight or branched chain alkynyl.

24. The compound of embodiment 1, wherein one of R_1 and R_2 is fluoro, and the other is $-COR_x$, and wherein R_x selected from the group consisting of C_1 to C_6 straight or branched chain alkyl, C_1 to C_6 straight or branched chain alkenyl, and C_1 to C_6 straight or branched chain alkynyl.

25. The compound of embodiment 1, wherein one of R_1 and R_2 is halo, and the other is $-C_1$ to C_6 straight or branched chain alkyl.

26. The compound of embodiment 1, wherein one of R_1 and R_2 is fluoro, and the other is -C_1 to C_6 straight or branched chain alkyl.

27. The compound of embodiment 1, wherein one of R_1 and R_2 is halo, and the other is -C_1 to C_6 straight or branched chain alkenyl.

28. The compound of embodiment 1, wherein one of R_1 and R_2 is fluoro, and the other is -C_1 to C_6 straight or branched chain alkenyl.

29. The compound of embodiment 1, wherein one of R_1 and R_2 is halo, and the other is -C_1 to C_6 straight or branched chain alkoxy.

30. The compound of embodiment 1, wherein one of R_1 and R_2 is fluoro, and the other is -C_1 to C_6 straight or branched chain alkoxy.

31. The compound of embodiment 1, wherein one of R_1 and R_2 is halo, and the other is -C_1 to C_6 straight or branched chain alkenoxy.

32. The compound of embodiment 1, wherein one of R_1 and R_2 is fluoro, and the other is -C_1 to C_6 straight or branched chain alkenoxy.

33. The compound of embodiment 1, wherein at least one of R_1 and R_2 is -C_1 to C_6 straight or branched chain alkyl substituted with halo.

34. The compound of embodiment 1, wherein one of R_1 and R_2 is -C_1 to C_6 straight or branched chain alkyl substituted with halo, and the other is -H.

35. The compound of embodiment 1, wherein one of R_1 and R_2 is -C_1 to C_6 straight or branched chain alkyl substituted with fluoro, and the other is -H.

36. The compound of embodiment 1, wherein at least one of R_1 and R_2 is -C_1 to C_6 straight or branched chain alkenyl substituted with halo.

37. The compound of embodiment 1, wherein one of R_1 and R_2 is -C_1 to C_6 straight or branched chain alkenyl substituted with halo, and the other is -H.

38. The compound of embodiment 1, wherein one of R_1 and R_2 is -C_1 to C_6 straight or branched chain alkenyl substituted with fluoro, and the other is -H.

39. The compound of embodiment 1, wherein at least one of R_1 and R_2 is/are -C_1 to C_6 straight or branched chain alkoxy substituted with halo

40. The compound of embodiment 1, wherein one of R_1 and R_2 is -C_1 to C_6 straight or branched chain alkoxy substituted with halo, and the other is -H.

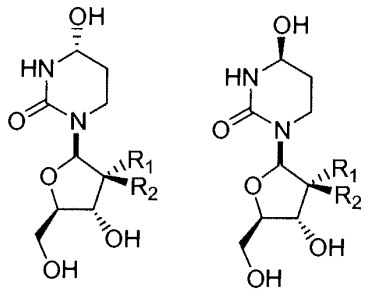
41. The compound of embodiment 1, wherein one of R_1 and R_2 is -C_1 to C_6 straight or branched chain alkoxy substituted with fluoro, and the other is -H.

42. The compound of embodiment 1, wherein at least one of R_1 and R_2 is -C_1 to C_6 straight or branched chain alkenoxy substituted with halo.

43. The compound of embodiment 1, wherein one of R_1 and R_2 is -C_1 to C_6 straight or branched chain alkenoxy substituted with halo, and the other is -H.

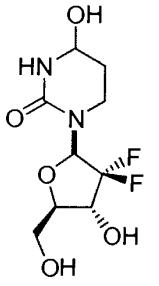
44. The compound of embodiment 1, wherein one of R_1 and R_2 is -C_1 to C_6 straight or branched chain alkenoxy substituted with fluoro, and the other is -H.

45. The compound of any one of embodiments 1 to 44, wherein the compound of Formula I has the stereochemistry of either Ia or Ib:

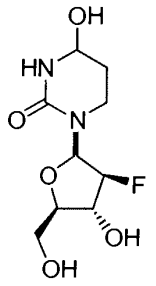


Ia Ib

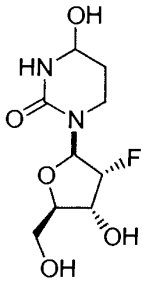
46. The compound of embodiment 1, wherein the compound is selected from Compounds 1 to 23:



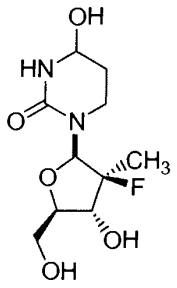
Compound 1



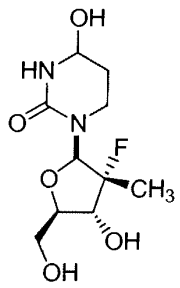
Compound 2



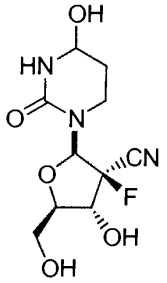
Compound 3



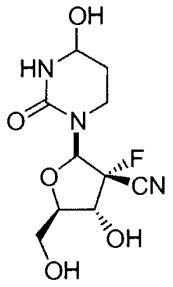
Compound 4



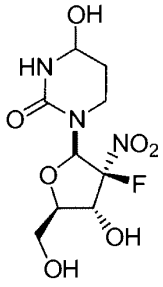
Compound 5



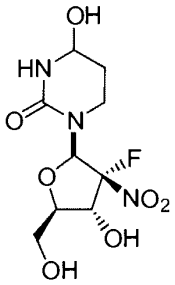
Compound 6



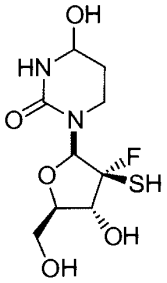
Compound 7



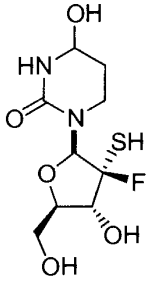
Compound 8



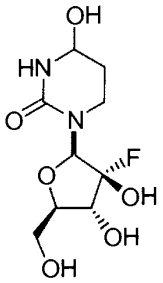
Compound 9



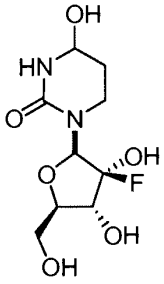
Compound 10



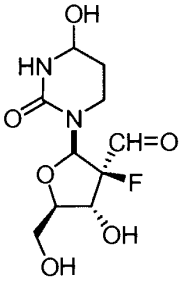
Compound 11



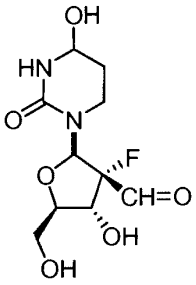
Compound 12



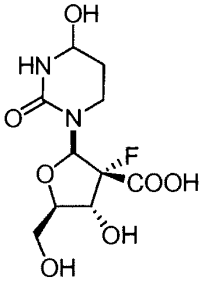
Compound 13



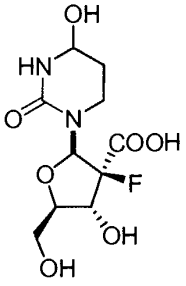
Compound 14



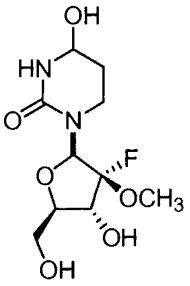
Compound 15



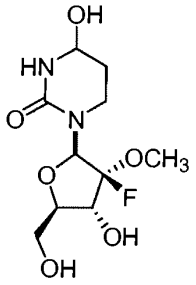
Compound 16



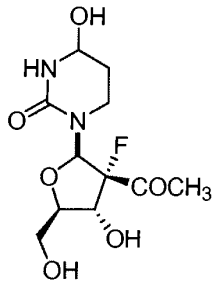
Compound 17



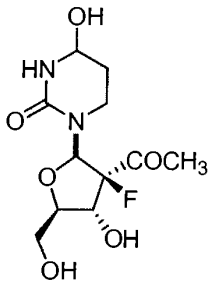
Compound 18



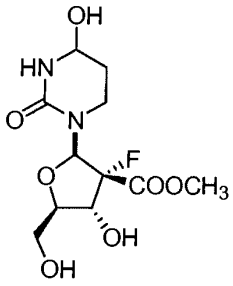
Compound 19



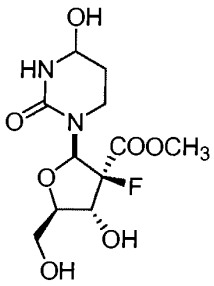
Compound 20



Compound 21

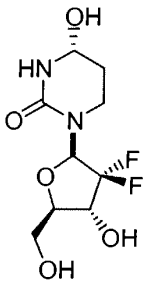


Compound 22

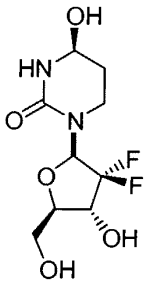


Compound 23

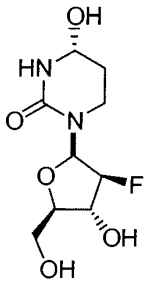
47. The compound of embodiment 1, wherein the compound is selected from the group consisting of 1a, 1b, 2a, 2b, 3a, and 3c:



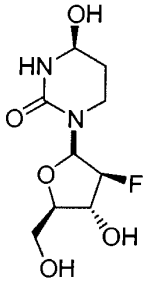
Compound 1a



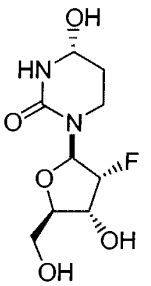
Compound 1b



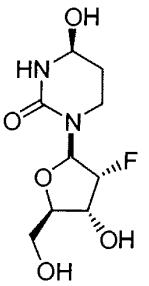
Compound 2a



Compound 2b



Compound 3a



Compound 3b

48. A pharmaceutical composition comprising:

(i) an effective amount of a compound of any one of embodiments 1 to 47 or a pharmaceutically acceptable salt thereof; and

(ii) a pharmaceutically acceptable excipient.

49. A method for inhibiting cytidine deaminase, comprising administering to a subject in need thereof an effective amount of a compound or pharmaceutical composition of any one of embodiments 1 to 47.

50. A method for treating cancer comprising administering to a subject in need thereof:

(i) an effective amount of a compound or pharmaceutical composition of any one of embodiments 1 to 48; and

(ii) a CDA substrate drug.

51. A method for inhibiting degradation of a CDA substrate drug by cytidine deaminase, comprising administering an effective amount of a compound or pharmaceutical composition of any one of embodiments 1 to 48 to a subject that is undergoing treatment with the CDA substrate drug.

52. The method of any one of embodiments 49 to 51, wherein the CDA substrate drug is selected from decitabine, 5-azacytidine, gemcitabine, ara-C, troxacitabine, tezacitabine, 5'-fluoro-2'-deoxycytidine, and cytochlor.

53. The method of any one of embodiments 49 to 52, wherein the compound of claim 1 is administered prior to the CDA substrate drug.

54. The method of any one of embodiments 49 to 52, wherein the compound of claim 1 is administered at substantially the same time with the CDA substrate drug.

55. The method of any one of embodiments 49 to 52, wherein the compound of claim 1 is administered after the CDA substrate drug.

56. The method of any one of embodiments 49 to 55, wherein the subject is a mammal.

57. The method of any one of embodiments 49 to 55, wherein the subject is a human.

58. The method of any one of embodiments 50 and 52 to 57, wherein the cancer is selected from hematological cancers and solid cancers.

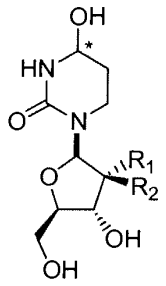
59. The method of any one of embodiments 50 and 52 to 57, wherein the cancer is a hematological cancer selected from MDS and leukemia.

60. The method of embodiment 59, wherein the leukemia is AML or CML.

61. The method of embodiment 58, where the cancer is a solid cancer selected from pancreatic cancer, ovarian cancer, peritoneal cancer, non small cell lung cancer, and breast cancer.

62. A kit comprising at least one unit dosage form, wherein the unit dosage form comprises a compound or pharmaceutical composition of any one of embodiments 1 to 48.

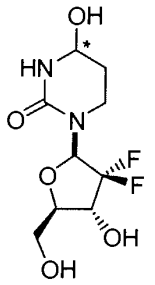
63. A compound of Formula I, or a pharmaceutically acceptable salt thereof:



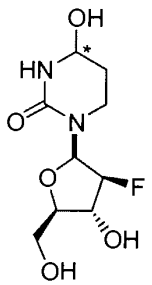
I

wherein the carbon marked by an asterisk may have an (R) or an (S) configuration; and wherein R_1 and R_2 are independently selected from fluoro and hydrogen, with the proviso that R_1 and R_2 may not both be hydrogen.

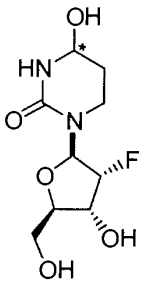
64. A compound of Formula I selected from the group consisting of



Compound 1



Compound 2

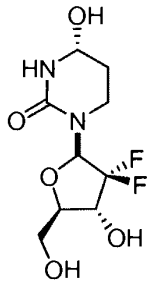


Compound 3

wherein each carbon atom marked with a * has a stereochemical configuration selected from the group consisting of an (R) configuration, an (S) configuration or a mixture of (R) and (S) configurations; and pharmaceutically acceptable salts thereof.

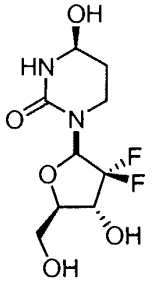
65. The compound of embodiment 64, wherein the compound is Compound 1.

66. The compound of embodiment 65, wherein Compound 1 has a stereochemistry represented by Compound 1a:



Compound 1a.

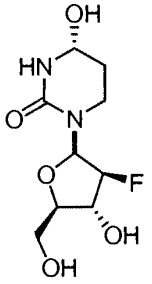
67. The compound of embodiment 65, wherein Compound 1 has a stereochemistry represented by Compound 1b:



Compound 1b.

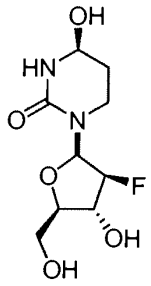
68. The compound of embodiment 64, wherein the compound is Compound 2.

69. The compound of embodiment 68, wherein Compound 2 has a stereochemistry represented by Compound 2a:



Compound 2a.

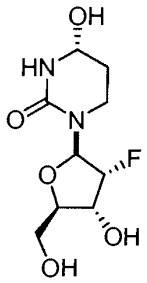
70. The compound of embodiment 68, wherein Compound 2 has a stereochemistry represented by Compound 2b:



Compound 2b

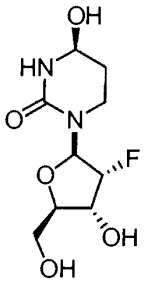
71. The compound of embodiment 64, wherein the compound is Compound 3.

72. The compound of embodiment 71, wherein Compound 3 has a stereochemistry represented by Compound 3a:



Compound 3a

73. The compound of embodiment 71, wherein Compound 3 has a stereochemistry represented by Compound 3b:



Compound 3b

74. A pharmaceutical composition comprising a compound of embodiment 64 or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable excipient.

75. The pharmaceutical composition of embodiment 74, further comprising a CDA substrate drug.

76. The pharmaceutical composition of embodiment 75, wherein the CDA substrate drug is selected from the group consisting of decitabine, 5-azacytidine, gemcitabine, ara-C, troxacitabine, tezacitabine, 5'-fluoro-2'-deoxycytidine, and cytochlor.

77. A method for treating cancer, comprising the steps of:

(i) administering to a mammal in need thereof a first pharmaceutical composition comprising an effective amount of a compound of embodiment 64; and

(ii) administering to a mammal in need thereof a second pharmaceutical composition comprising a CDA substrate drug.

78. The method of embodiment 77, wherein the CDA substrate drug is selected from the group consisting of decitabine, 5-azacytidine, gemcitabine, ara-C, troxacitabine, tezacitabine, 5'-fluoro-2'-deoxycytidine, and cytochlor.

79. The method of embodiment 77, wherein said cancer is a cancer being treated with a CDA substrate drug.

80. The method of embodiment 77, wherein the cancer is selected from the group consisting of hematological cancers and solid cancers.

81. The method of embodiment 80, wherein the cancer is a hematological cancer selected from the group consisting of myelodysplastic syndrome and leukemia.

82. The method of embodiment 81, wherein the leukemia is acute myeloid leukemia or chronic myeloid leukemia.

83. The method of embodiment 80, where the cancer is a solid cancer selected from the group consisting of pancreatic cancer, ovarian cancer, peritoneal cancer, non small cell lung cancer, and metastatic breast cancer.

84. A method for inhibiting degradation of a CDA substrate drug by cytidine deaminase, comprising administering an effective amount of a pharmaceutical composition comprising a compound of embodiment 64 to a mammal that is undergoing treatment with said CDA substrate drug.

85. The method of embodiment 84 wherein the CDA substrate drug is selected from the group consisting of decitabine, 5-azacytidine, gemcitabine, ara-C, troxacitabine, tezacitabine, 5'-fluoro-2'-deoxycytidine, and cytochlor.

86. A compound of embodiment 64 for use in inhibiting cytidine deaminase in a subject in need thereof.

87. Use of a compound of embodiment 64 for the manufacture of a medicament for inhibiting cytidine deaminase in a subject in need thereof.

88. A compound of embodiment 64 for use in treating cancer in a subject being treated with a CDA substrate drug.

89. Use of a compound of embodiment 64 for the manufacture of a medicament for treating cancer in a subject being treated with a CDA substrate drug.

90. A compound of embodiment 64 for use in inhibiting degradation of a CDA substrate drug by cytidine deaminase in a mammal that is undergoing treatment with the CDA substrate drug.

91. Use of a compound of embodiment 64 for the manufacture of a medicament for inhibiting degradation of a CDA substrate drug by cytidine deaminase.

92. The method of embodiment 77, wherein the compound of embodiment 64 is administered prior to the CDA substrate drug.

93. The method of embodiment 77, wherein the compound of embodiment 64 is administered at substantially the same time with the CDA substrate drug.

94. The method of embodiment 77, wherein the compound of embodiment 64 is administered after the CDA substrate drug.

95. A pharmaceutical composition comprising a compound of claim 2 or a pharmaceutically acceptable salt thereof, and decitabine; and a pharmaceutically acceptable excipient.

96. A method for treating cancer, comprising the steps of:

(i) administering to a mammal in need thereof a first pharmaceutical composition comprising an effective amount of a compound of claim 2; and

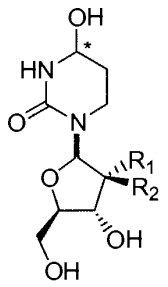
(ii) administering to a mammal in need thereof a second pharmaceutical composition comprising decitabine.

While the present invention has been described with reference to the specific embodiments thereof, it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the true spirit and scope of the invention. In addition, many modifications may be made to adapt a particular situation, material, composition of matter, process, process step or steps, to the spirit and scope of the present invention. All such modifications are intended to be within the scope of the claims appended hereto.

All patents and publications cited above are hereby incorporated by reference.

WHAT IS CLAIMED IS:

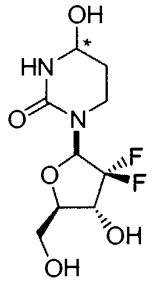
1. A compound of Formula I, or a pharmaceutically acceptable salt thereof:



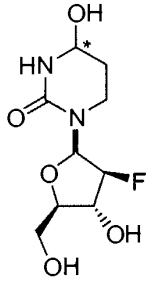
I

wherein the carbon marked by an asterisk may have an (R) or an (S) configuration; and wherein R_1 and R_2 are independently selected from fluoro and hydrogen, with the proviso that R_1 and R_2 may not both be hydrogen.

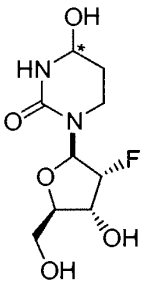
2. A compound of Formula I selected from the group consisting of



Compound 1



Compound 2

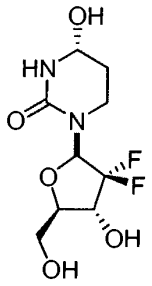


Compound 3

wherein each carbon atom marked with a * has a stereochemical configuration selected from the group consisting of an (R) configuration, an (S) configuration or a mixture of (R) and (S) configurations; and pharmaceutically acceptable salts thereof.

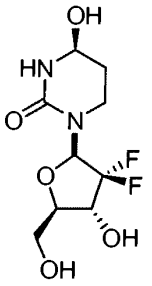
3. The compound of claim 2, wherein the compound is Compound 1.

4. The compound of claim 3, wherein Compound 1 has a stereochemistry represented by Compound 1a:



Compound 1a

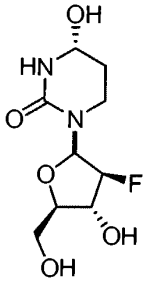
5. The compound of claim 2, wherein Compound 1 has a stereochemistry represented by Compound 1b:



Compound 1b

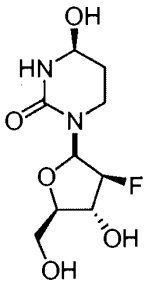
6. The compound of claim 2, wherein the compound is Compound 2.

7. The compound of claim 6, wherein Compound 2 has a stereochemistry represented by Compound 2a:



Compound 2a

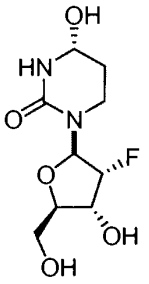
8. The compound of claim 6, wherein Compound 2 has a stereochemistry represented by Compound 2b:



Compound 2b

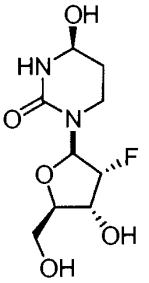
9. The compound of claim 2, wherein the compound is Compound 3.

10. The compound of claim 9, wherein Compound 3 has a stereochemistry represented by Compound 3a:



Compound 3a

11. The compound of claim 9, wherein Compound 3 has a stereochemistry represented by Compound 3b:



Compound 3b

12. A pharmaceutical composition comprising a compound of claim 2 or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable excipient.

13. The pharmaceutical composition of claim 12, further comprising a CDA substrate drug.

14. The pharmaceutical composition of claim 13, wherein the CDA substrate drug is selected from the group consisting of decitabine, 5-azacytidine, gemcitabine, ara-C, troxacitabine, tezacitabine, 5'-fluoro-2'-deoxycytidine, and cytochlor.

15. A pharmaceutical composition comprising a compound of claim 2 or a pharmaceutically acceptable salt thereof, and decitabine; and a pharmaceutically acceptable excipient.

16. A method for treating cancer, comprising the steps of:

(i) administering to a mammal in need thereof a first pharmaceutical composition comprising an effective amount of a compound of claim 2; and

(ii) administering to a mammal in need thereof a second pharmaceutical composition comprising a CDA substrate drug.

17. The method of claim 16, wherein the CDA substrate drug is selected from the group consisting of decitabine, 5-azacytidine, gemcitabine, ara-C, troxacitabine, tezacitabine, 5'-fluoro-2'-deoxycytidine, and cytochlor.

18. A method for treating cancer, comprising the steps of:

(i) administering to a mammal in need thereof a first pharmaceutical composition comprising an effective amount of a compound of claim 2; and

(ii) administering to a mammal in need thereof a second pharmaceutical composition comprising decitabine.

19. The method of claim 17, wherein said cancer is a cancer being treated with a CDA substrate drug.

20. The method of claim 17, wherein the cancer is selected from the group consisting of hematological cancers and solid cancers.

21. The method of claim 20, wherein the cancer is a hematological cancer selected from the group consisting of myelodysplastic syndrome and leukemia.

22. The method of claim 21, wherein the leukemia is acute myeloid leukemia or chronic myeloid leukemia.

23. The method of claim 20, where the cancer is a solid cancer selected from the group consisting of pancreatic cancer, ovarian cancer, peritoneal cancer, non small cell lung cancer, and metastatic breast cancer.

24. A method for inhibiting degradation of a CDA substrate drug by cytidine deaminase, comprising administering an effective amount of a pharmaceutical composition comprising a compound of claim 2 to a mammal that is undergoing treatment with said CDA substrate drug.

25. The method of claim 24 wherein the CDA substrate drug is selected from the group consisting of decitabine, 5-azacytidine, gemcitabine, ara-C, troxacitabine, tezacitabine, 5'-fluoro-2'-deoxycytidine, and cytochlor.

26. A compound of claim 2 for use in inhibiting cytidine deaminase in a subject in need thereof.

27. Use of a compound of claim 2 for the manufacture of a medicament for inhibiting cytidine deaminase in a subject in need thereof.

28. A compound of claim 2 for use in treating cancer in a subject being treated with a CDA substrate drug.

29. Use of a compound of claim 2 for the manufacture of a medicament for treating cancer in a subject being treated with a CDA substrate drug.

30. A compound of claim 2 for use in inhibiting degradation of a CDA substrate drug by cytidine deaminase in a mammal that is undergoing treatment with the CDA substrate drug.

31. Use of a compound of claim 2 for the manufacture of a medicament for inhibiting degradation of a CDA substrate drug by cytidine deaminase.

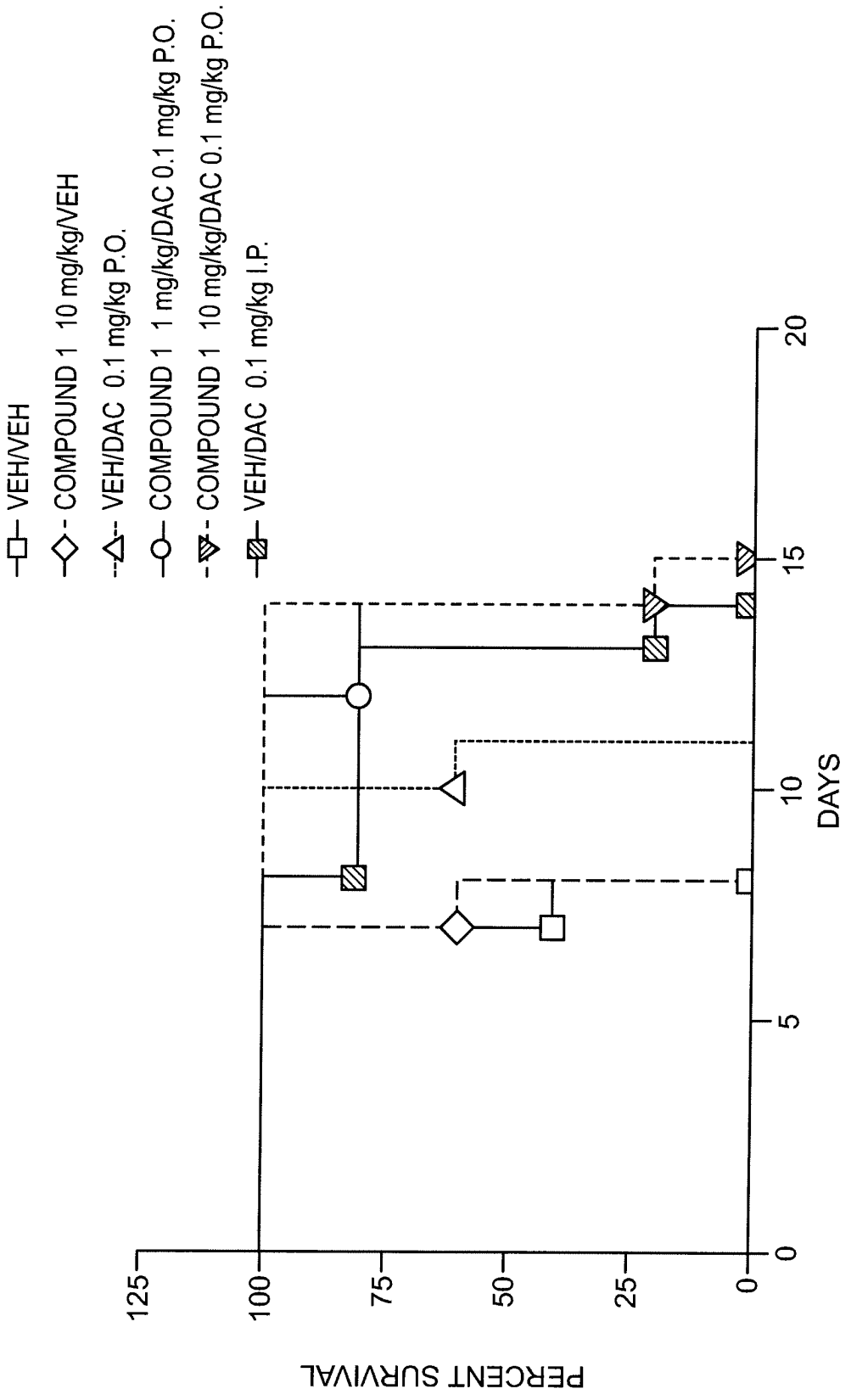


FIG. 1

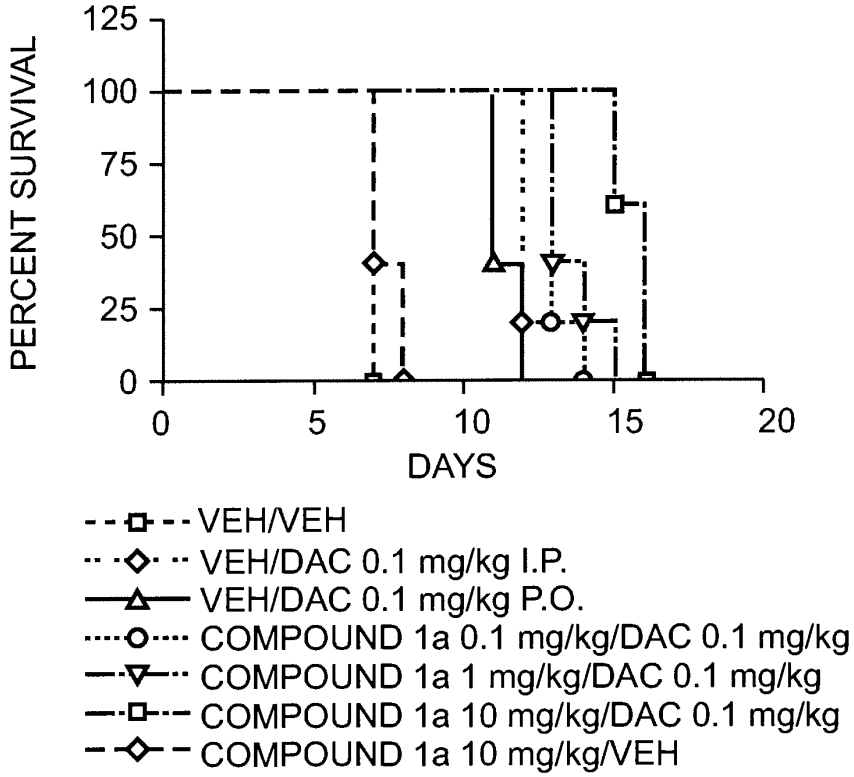

PERCENT SURVIVAL
125
100
75
50
25
0
0
5
10
15
20
DAYS
VEH/VEH
VEH/DAC 0.1 mg/kg I.P.
VEH/DAC 0.1 mg/kg P.O.
COMPOUND 1a 0.1 mg/kg/DAC 0.1 mg/kg
COMPOUND 1a 1 mg/kg/DAC 0.1 mg/kg
COMPOUND 1a 10 mg/kg/DAC 0.1 mg/kg
COMPOUND 1a 10 mg/kg/VEH

FIG. 2

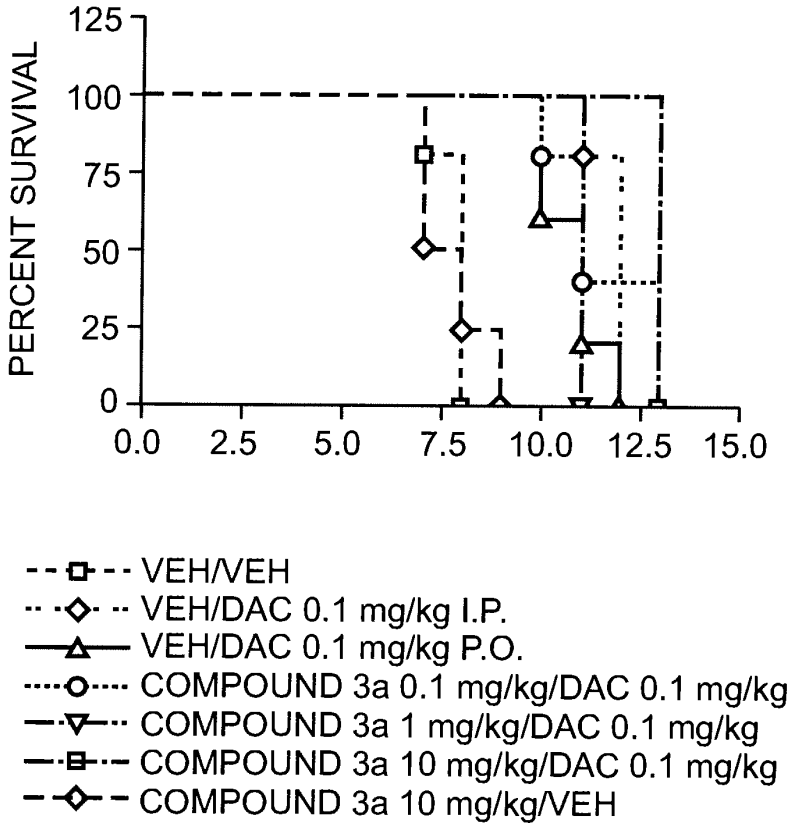


FIG. 3

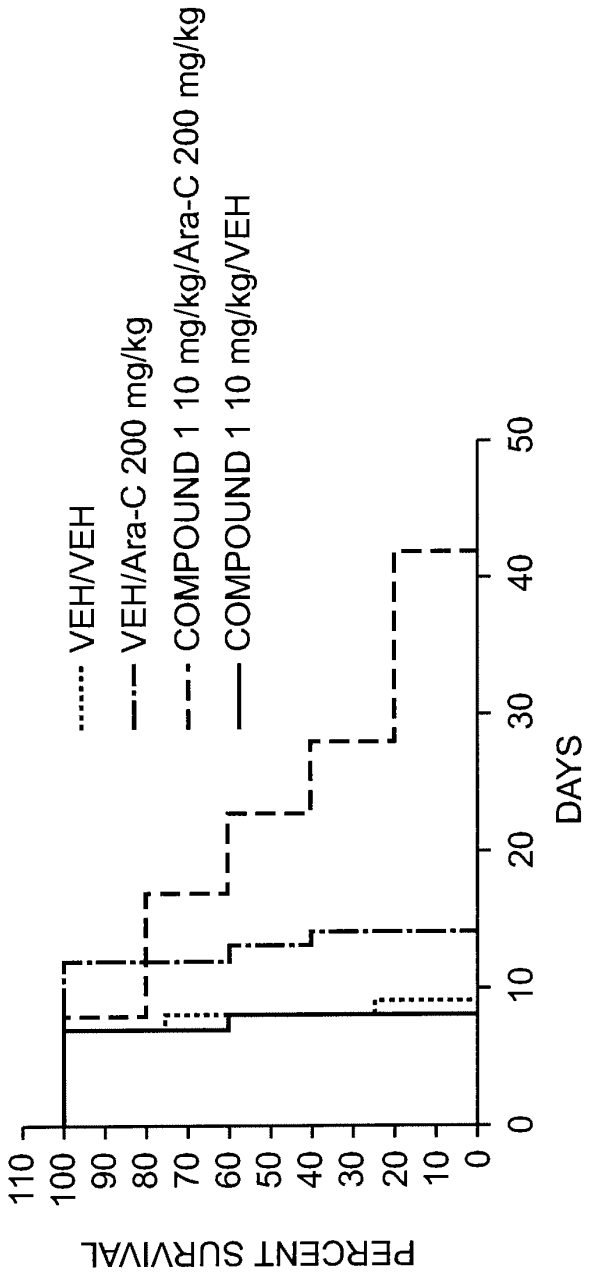

VEH/VEH
VEH/Ara-C 200 mg/kg
COMPOUND 1 10 mg/kg/Ara-C 200 mg/kg
COMPOUND 1 10 mg/kg/VEH
PERCENT SURVIVAL
110
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
DAYS

FIG. 4

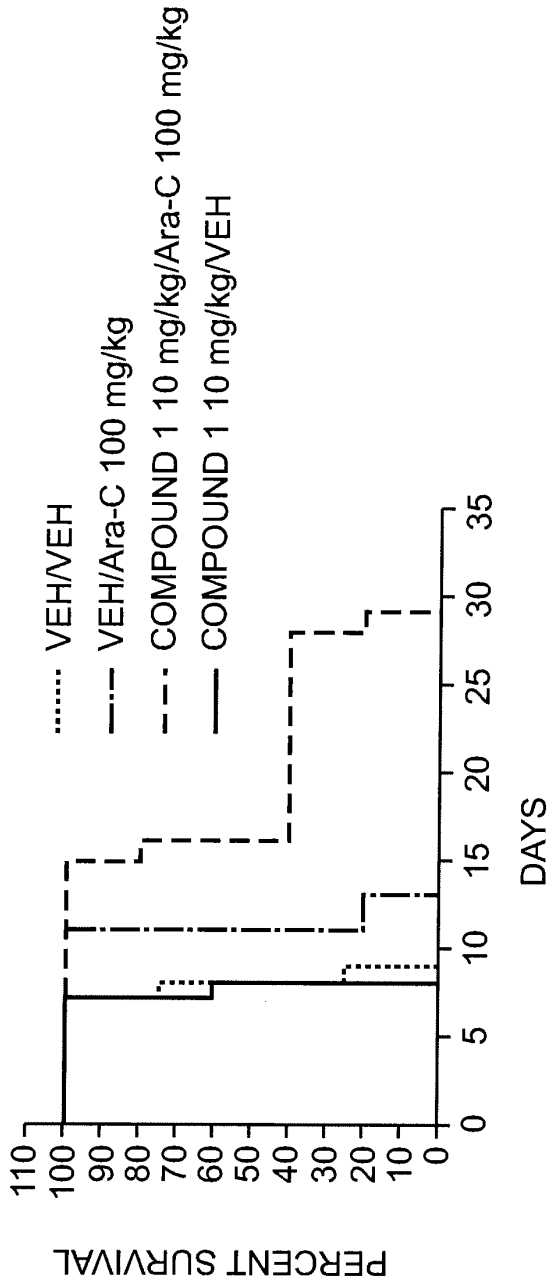

VEH/VEH
VEH/Ara-C 100 mg/kg
COMPOUND 1 10 mg/kg/Ara-C 100 mg/kg
COMPOUND 1 10 mg/kg/VEH
PERCENT SURVIVAL
110
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
DAYS

FIG. 5

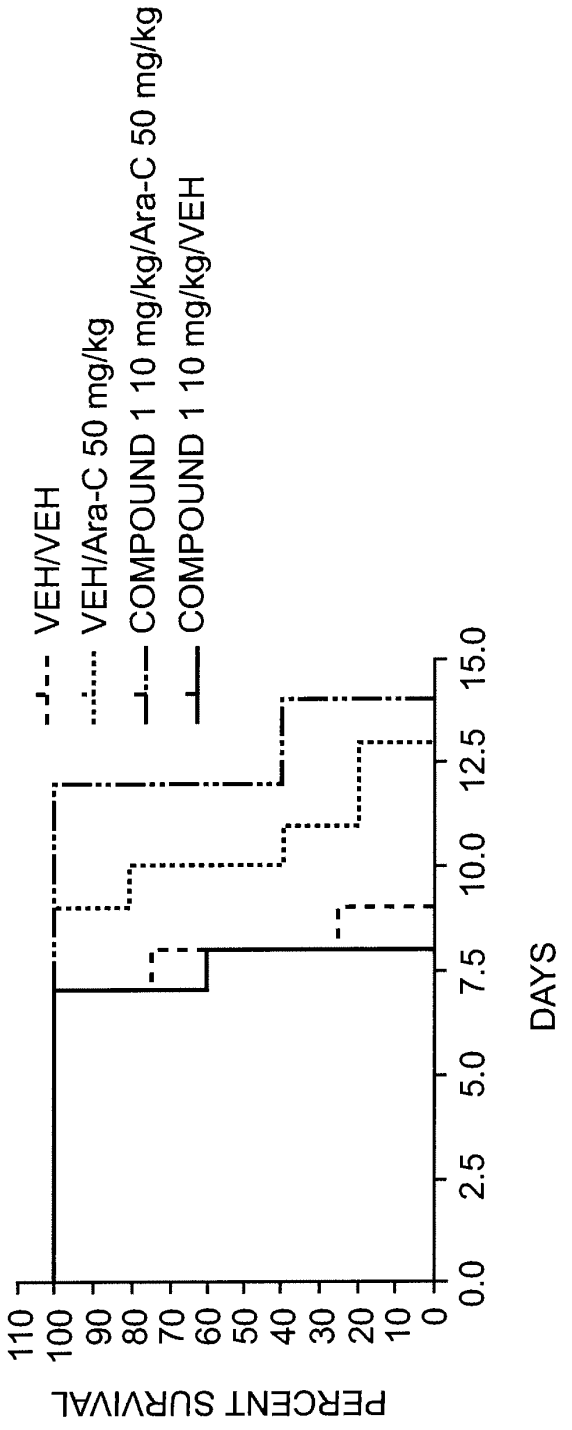

VEH/VEH
VEH/Ara-C 50 mg/kg
COMPOUND 1 10 mg/kg/Ara-C 50 mg/kg
COMPOUND 1 10 mg/kg/VEH
PERCENT SURVIVAL
110
100
90
80
70
60
50
40
30
20
10
0
0.0
2.5
5.0
7.5
10.0
12.5
15.0
DAYS

FIG. 6

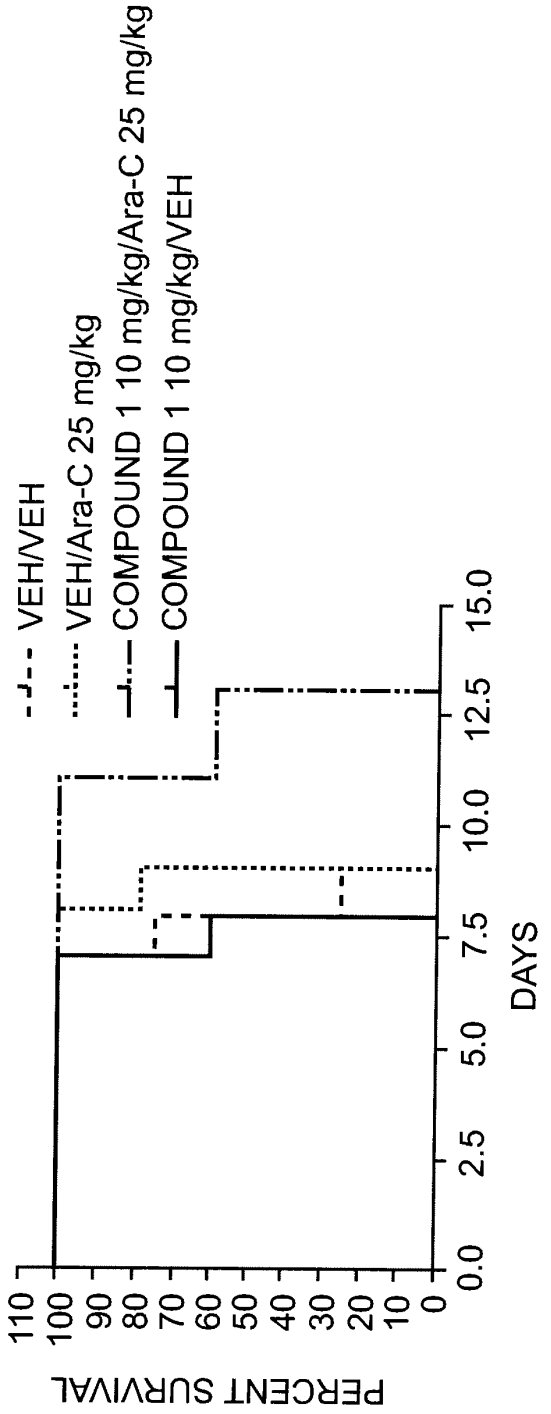

VEH/VEH
VEH/Ara-C 25 mg/kg
COMPOUND 1 10 mg/kg/Ara-C 25 mg/kg
COMPOUND 1 10 mg/kg/VEH
PERCENT SURVIVAL
110
100
90
80
70
60
50
40
30
20
10
0
0.0
2.5
5.0
7.5
10.0
12.5
15.0
DAYS

FIG. 7

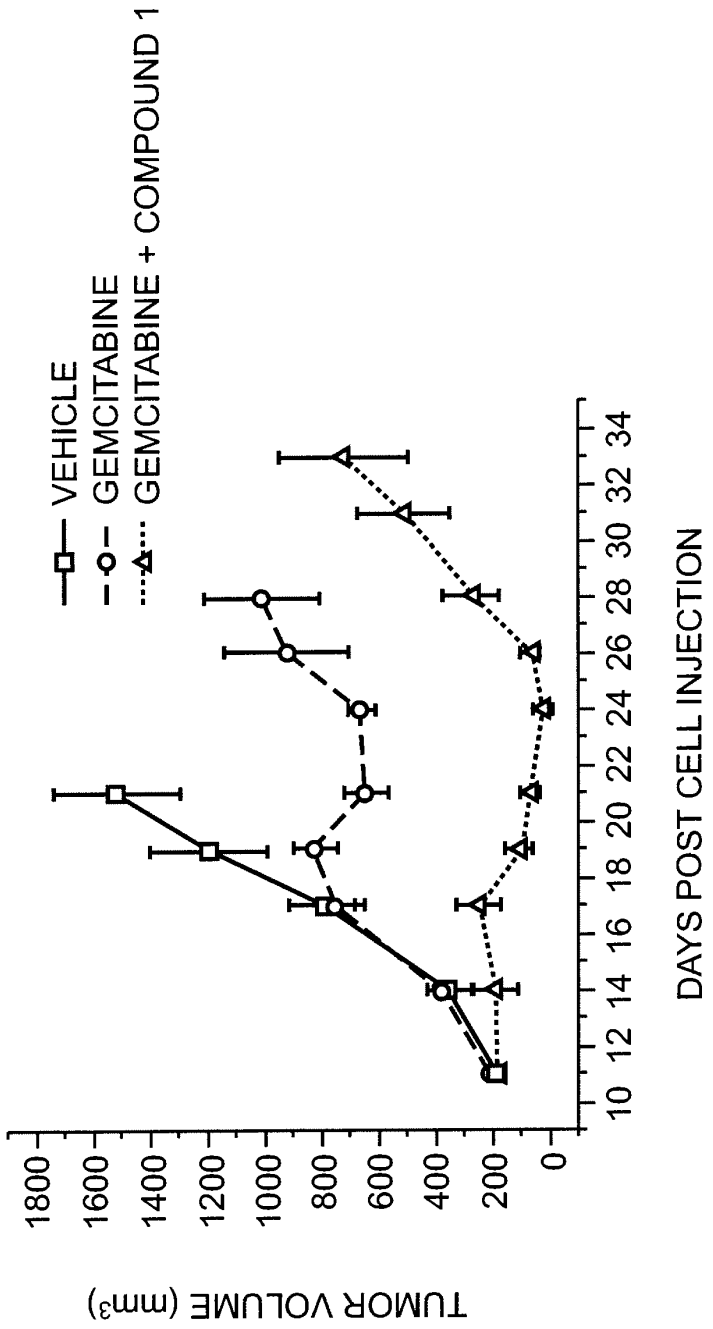

VEHICLE
GEMCITABINE
GEMCITABINE + COMPOUND 1
TUMOR VOLUME (mm3)
1800
1600
1400
1200
1000
800
600
400
200
0
10 12 14 16 18 20 22 24 26 28 30 32 34
DAYS POST CELL INJECTION

FIG. 8

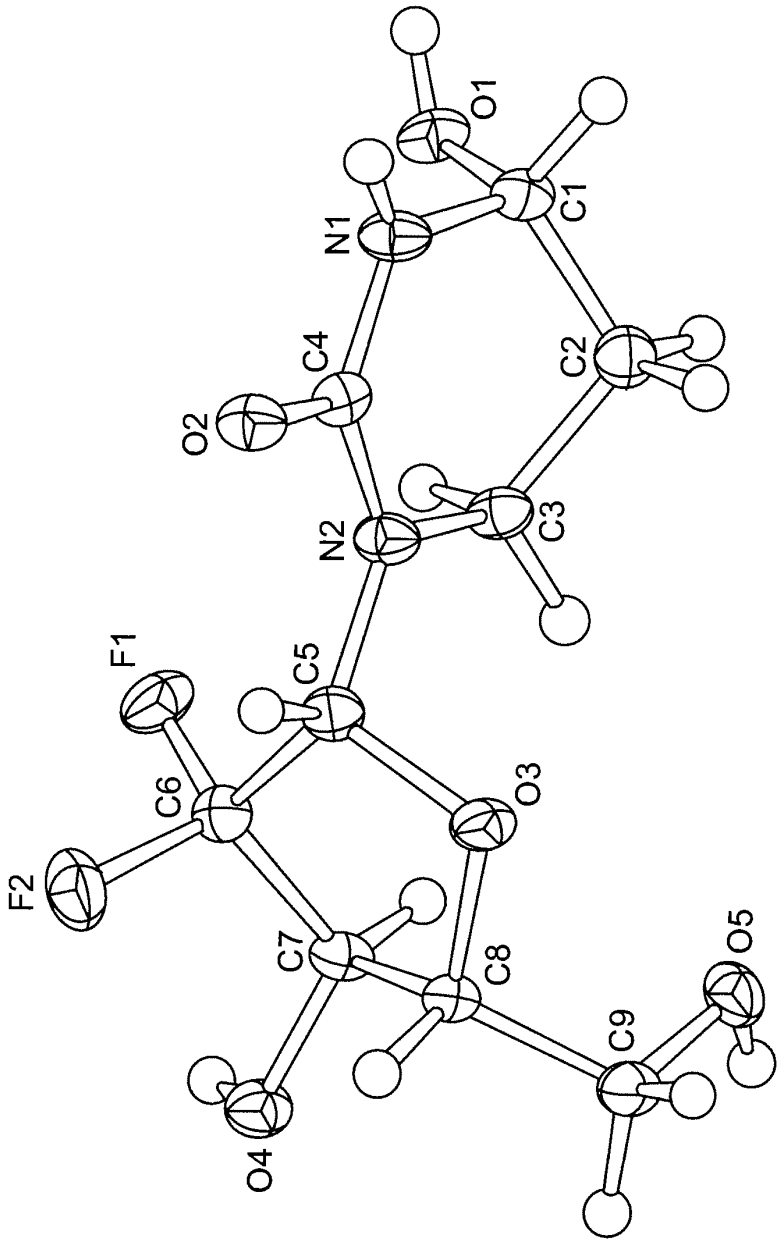
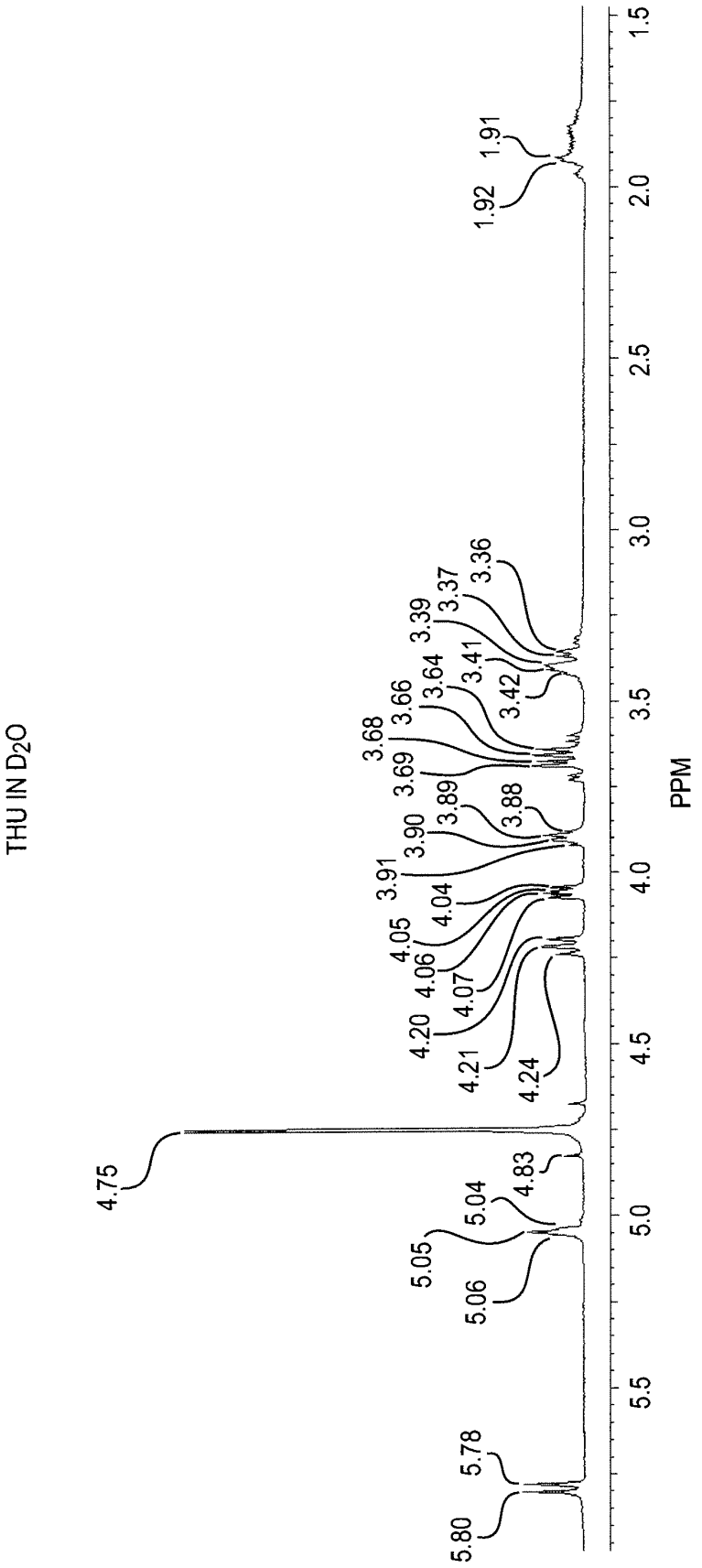


FIG. 9


THU IN D_2O
PPM
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
1.91
1.92
3.36
3.37
3.39
3.41
3.42
3.64
3.66
3.68
3.69
3.88
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3.91
4.04
4.05
4.06
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5.05
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FIG. 10

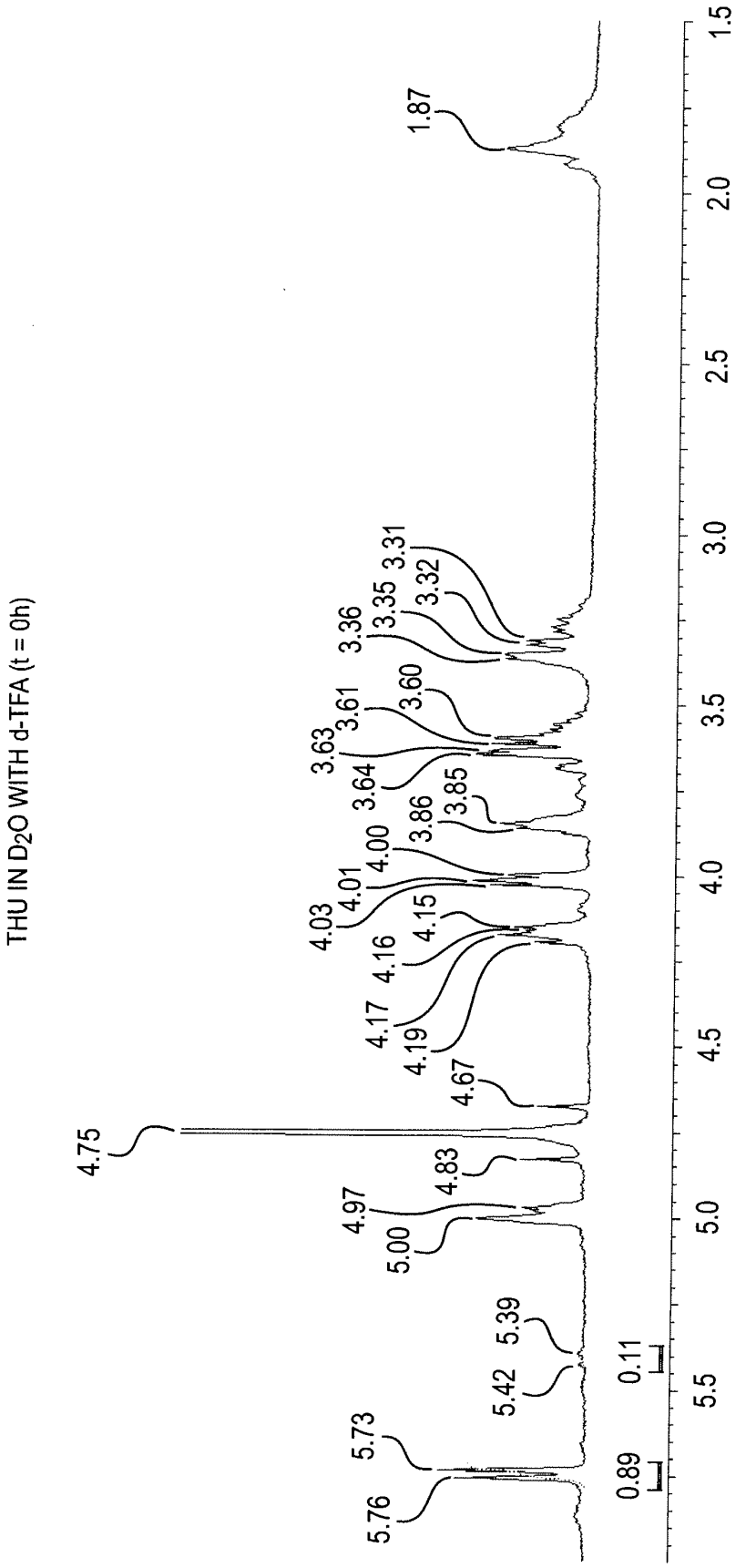


FIG. 11A

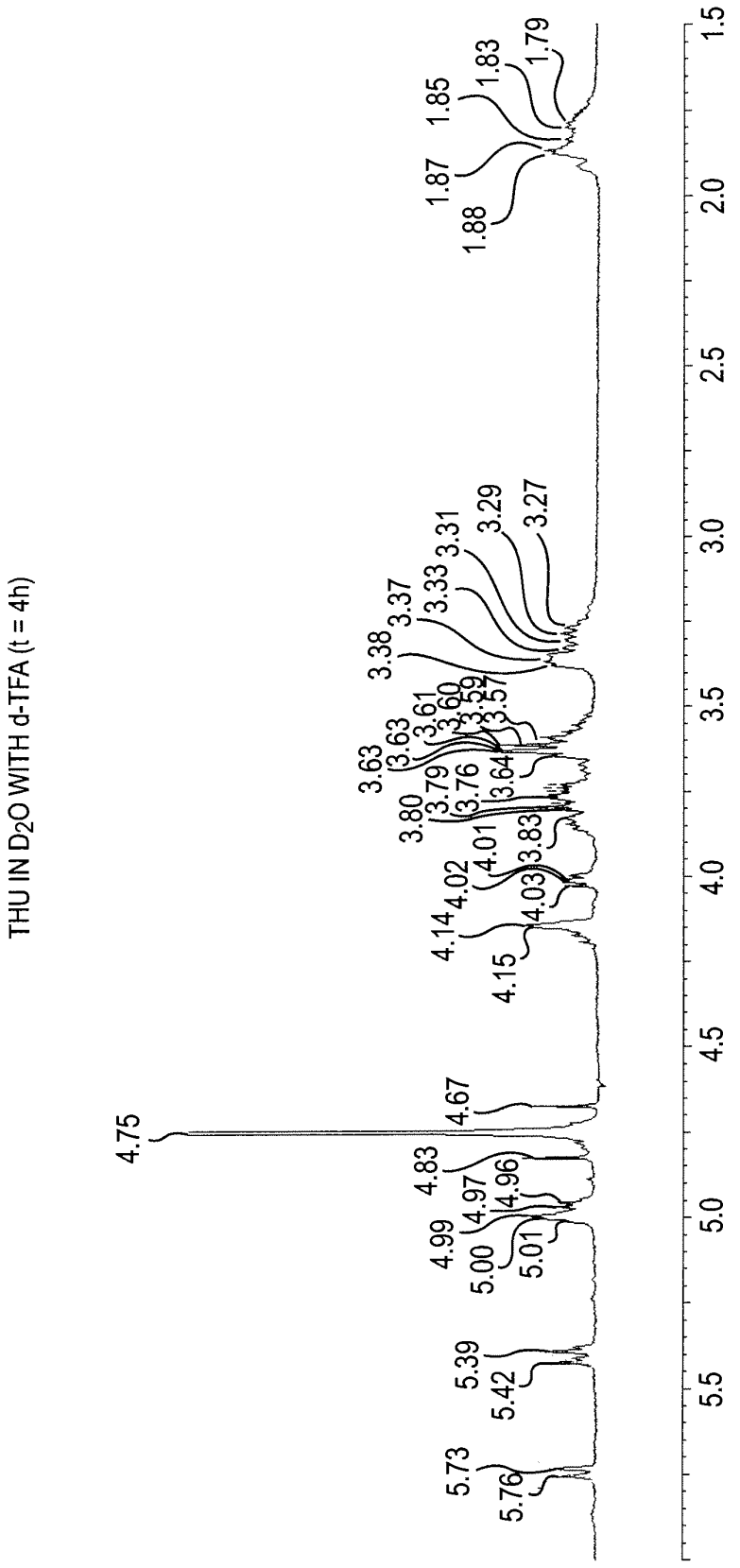


FIG. 11B

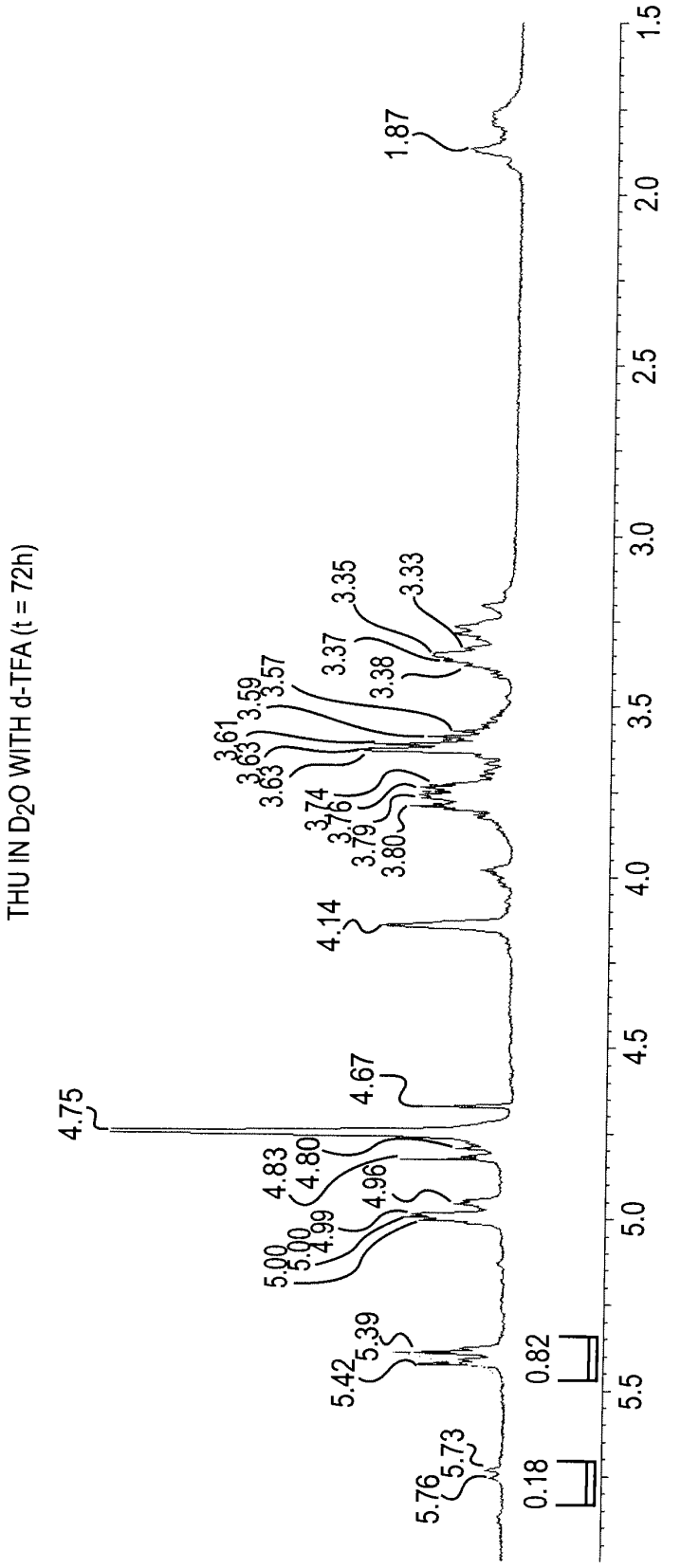
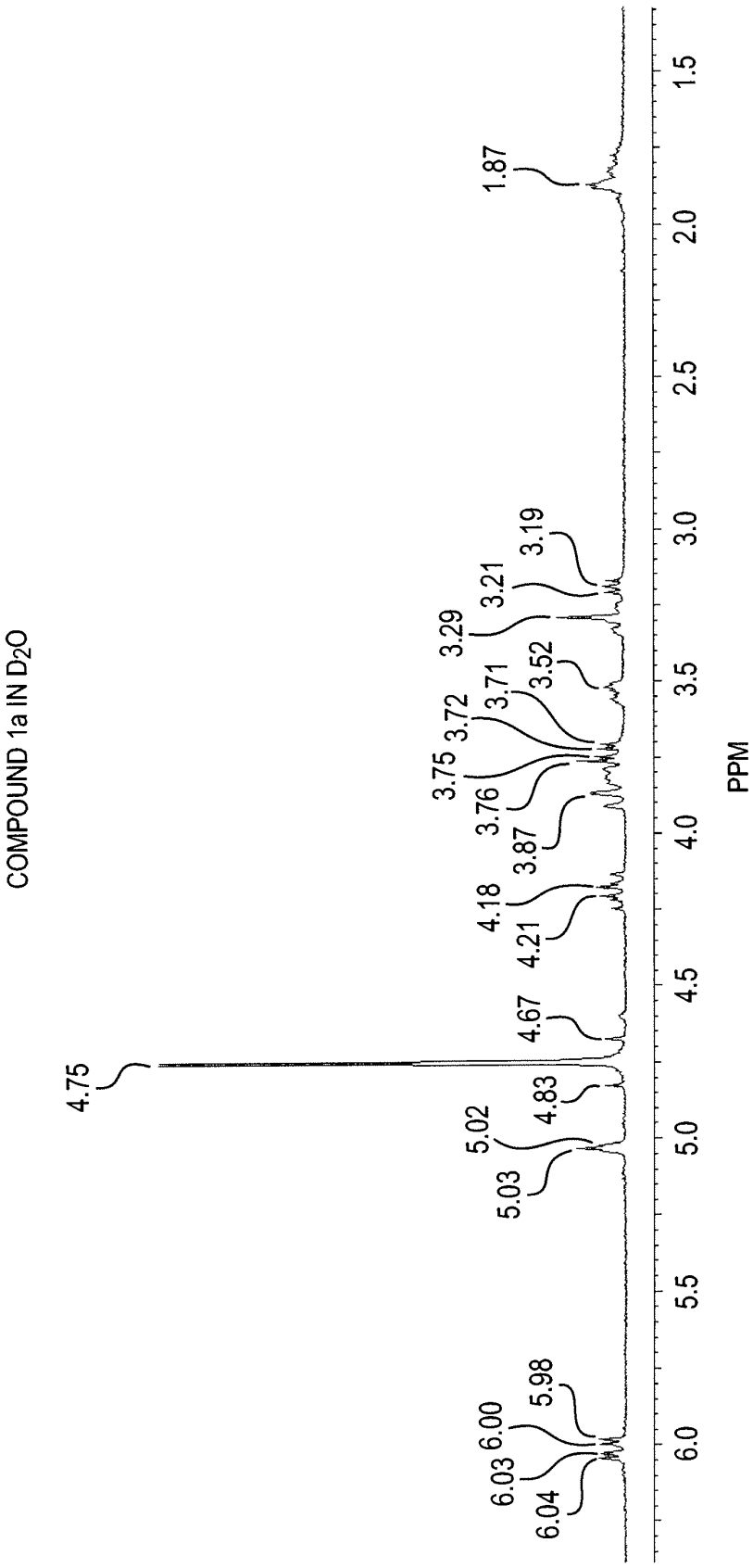


FIG. 11C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4.67
4.21
4.18
3.87
3.76
3.75
3.72
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3.52
3.29
3.21
3.19
1.87
5.02
4.83
5.03
6.03
6.00
5.98
6.04
6.0
5.5
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4.0
3.5
3.0
2.5
2.0
1.5
PPM

FIG. 12

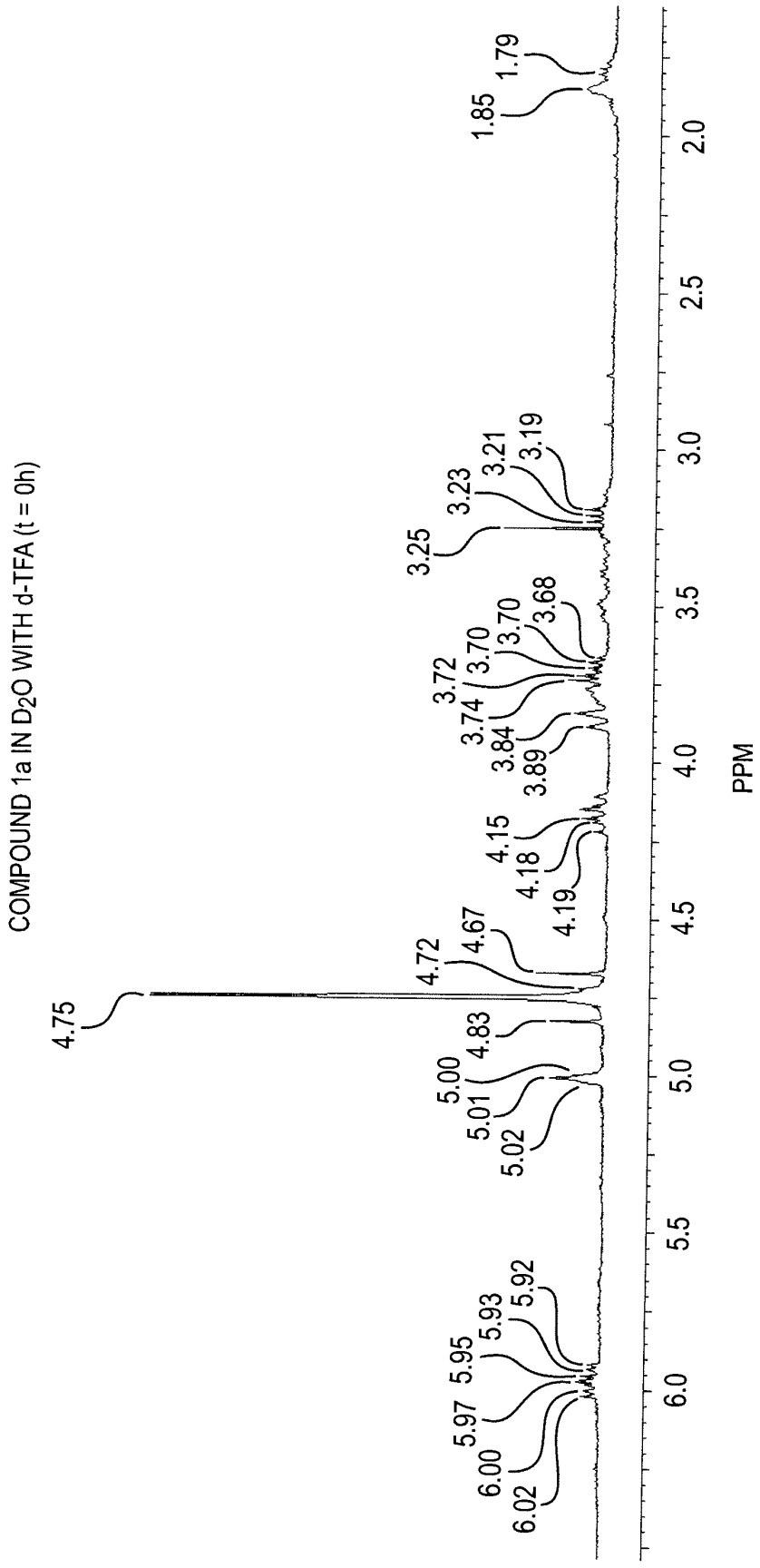
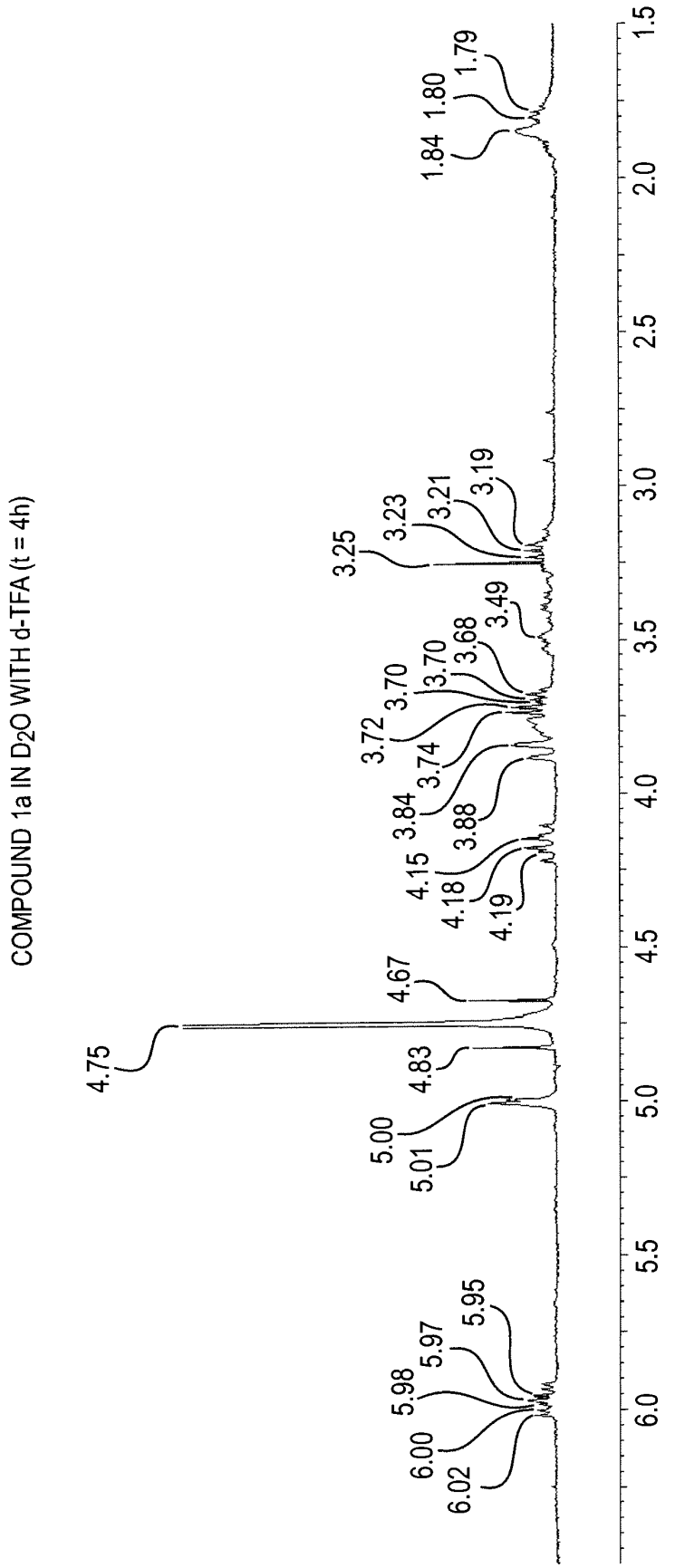


FIG. 13A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4.67
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5.00
5.01
6.00
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4.15
4.18
4.19
3.84
3.88
3.72
3.74
3.70
3.70
3.68
3.49
3.25
3.23
3.21
3.19
1.84
1.80
1.79
PPM
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4.0
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2.0
1.5

FIG. 13B

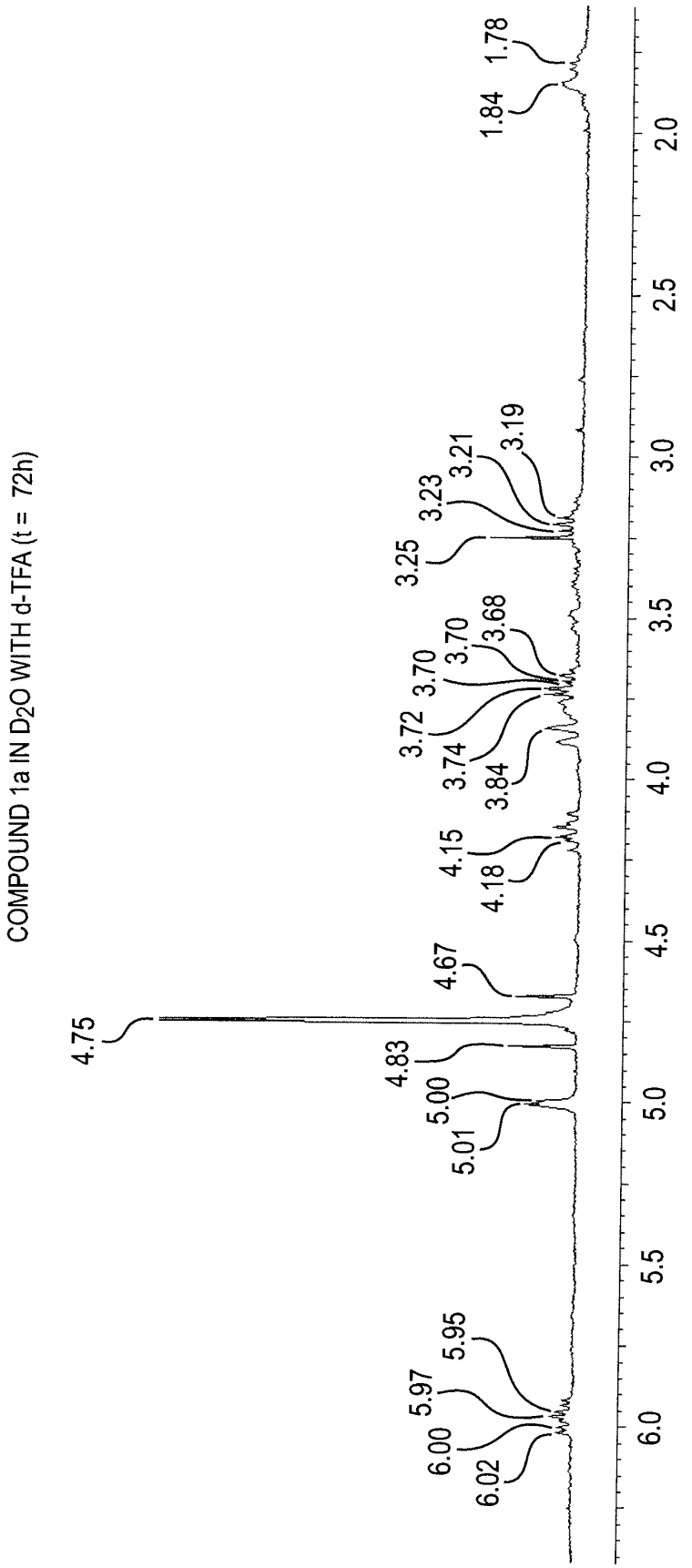

FIG. 13C

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2008/080163

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07H19/04 A61K31/706 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)
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 practical, search terms used)
EPO-Internal, CHEM ABS Data, BEILSTEIN Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 2008/085611 A (UNIVERSITY OF MIAMI) 17 July 2008 (2008-07-17) claim 28	1-31
A	K. A. WATANABE ET AL.: "Nucleosides. 110. Synthesis and Antiherpes Virus Activity of Some 2'-Fluoro-2'-deoxyarabinofuranosylpyrimidine Nucleosides" J. MED. CHEM., vol. 22, no. 1, 1979, pages 21-24, XP002511651 * Compound of formula 9a * table I	1-31
A	US 4 017 606 A (A. R. HANZE, G. W. CAMIENER) 12 April 1977 (1977-04-12) claims 1-5	1-31

☐ Further documents are listed in the continuation of Box C.

☒ 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
23 January 2009	11/02/2009

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	Herz, Claus

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

International application No
PCT/US2008/080163

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2008085611 A	17-07-2008	NONE	
US 4017606 A	12-04-1977	NONE	

Review of the clinical experience with 5-azacytidine and 5-aza-2'-deoxycytidine in solid tumors

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In recent years the importance of epigenetic changes in carcinogenesis has been unfolding. It is now clear that the fifth base of the genome, methylcytosine, plays a critical role in the control of gene expression during normal development and carcinogenesis. Efforts to decrease methylation in neoplasias as a therapeutic strategy have been productive in hematologic malignancies but disappointing in solid tumors. The following is a review of the clinical experience with the agents 5-azacytidine and 5-aza-2'-deoxycytidine in solid malignancies and a discussion of the difficulties encountered.

Keywords 5-Azacytidine, 5-aza-2-deoxycytidine, clinical trials, DNA methylation, solid tumor

DNA methylation

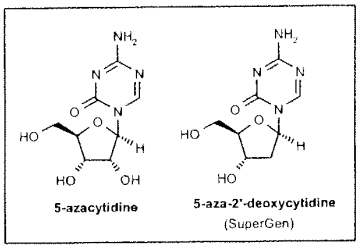
Methylation occurs after DNA replication by the addition of a methyl group from S-adenosylmethionine (SAM) to the 5'-position of cytosine residues. Approximately 3 to 4% of cytosines in mammalian DNA are methylated. Most of the 5-methylcytosine residues in eukaryotic DNA are found in the dinucleotide sequence 5'-CpG-3' [1•]. CpG dinucleotides are scarce throughout mammalian DNA, except in the so-called CpG islands, where their frequency is normal or higher than expected. Spontaneous deamination of 5-methylcytosine leads to thymine and thus methylated CpG sites are highly mutagenic. In fact, although CpG dinucleotides are only found at one-fifth of the expected frequency in human DNA, more than 30% of all known disease-related point mutations are found at these sites [2•,3•].

This raises the question of why the fifth base of the genome is maintained. The essential function of methylated cytosine residues appears to be to modify protein-DNA interactions and thereby suppress gene transcription. CpG islands are often located in the promoter regions of genes and it has been shown that methylation of their cytosine residues effectively switches off the downstream gene [4••]. Like genetic mutations, a methylation pattern is information that is stable and reproduced with each round of cell division, but unlike genetic mutations, it can be readily reversed. This property makes DNA methylation an essential tool during embryonic development. Shortly after fertilization, the methylation patterns observed in the mature oocytes and sperm are wiped out with a genome-wide wave of demethylation. Then, selective de novo methylation takes place, sparing the housekeeping genes and those that need to be active during embryogenesis. Finally, tissue-specific genes are demethylated in association with the onset of their activity, which presumably leads to tissue differentiation. DNA methylation is also involved in the inactivation of the X chromosome in females and in parental imprinting, and it probably contributes to the aging process [5•,6].

Changes in methylation are among the most common genomic alterations found in neoplasia. On one hand, there is global hypomethylation of the DNA leading to chromosomal instability and an increased rate of genetic mutations [7•]. On the other, there is hypermethylation of CpG islands located in the promoters of tumor suppressor genes such as *p16*, *p15*, *VHL* and *Rb*, that renders them silent and provides a growth advantage for the cell [8]. In this regard, it is interesting to note that the establishment of immortal cell lines *in vitro* is also associated with *de novo* methylation of CpG islands [9•,10]. Other genes known to contribute to tumorigenesis such as the DNA-repair gene *MLH1* [11], E-cadherin [12], cyclooxygenase-2 [13,14] and estrogen receptor α [15,16,17] are also silenced by methylation of CpG islands in their upstream promoters. Additionally, it appears that resistance to chemotherapeutic agents may be mediated by methylation of genes in the apoptotic pathway. For example, the restoration of *Apaf-1* expression in highly chemoresistant melanoma cell lines after treatment with 5-aza-2'-deoxycytidine, led to a marked enhancement in their sensitivity to adriamycin and a rescue of the apoptotic defects associated with *Apaf-1* silencing [18••]. If these epigenetic changes could be reversed, we would potentially be able to re-establish antiproliferation, differentiation and chemotherapy sensitivity pathways in malignant cells.

5-Azacytidine and 5-aza-2'-deoxycytidine (decitabine; SuperGen Inc; Figure 1) are pyrimidine analogs that result from substituting nitrogen at the fifth carbon position of the nucleosides cytidine and 2'-deoxycytidine, respectively. When they are incorporated into replicating DNA they form a covalent complex with DNA methyltransferase 1 (DNMT1, responsible for reproducing the methylation patterns in the daughter strands) and deplete the cell of this enzymatic activity leading to the synthesis of hypomethylated strands [19•]. At high concentrations these drugs are cytotoxic but at lower concentrations they induce differentiation of cell lines [20]. It is believed that the changes observed in cells treated with these drugs are a consequence of their induction of DNA demethylation and ensuing activation of silent genes, although some evidence suggests that covalent binding of the enzyme to the drug-substituted DNA is the primary mechanism of drug-induced toxicity [21•,22•]. Both compounds have been tested in clinical trials and found to have significant antitumor activity.

Figure 1. Structures of 5-azacytidine and 5-aza-2'-deoxycytidine.



Clinical experience in hematologic diseases

Both 5-azacytidine and 5-aza-2'-deoxycytidine, at doses ranging from 500 to 1500 mg/m^2, have proved effective in the treatment of relapsed or refractory acute leukemias and in the blast crisis of chronic myeloid leukemia. At low doses (50 to 150 mg/m^2) they have shown activity in myelodysplastic syndrome (MDS), leading to trilineage responses in some patients [23]. A randomized phase III trial of low-dose 5-azacytidine administered subcutaneously demonstrated a decreased probability of transformation to acute myeloid leukemia in high risk MDS patients, an improvement in quality of life and a trend towards improved survival [24]. There have been several reports of increase in fetal hemoglobin in patients with severe β-thalassemia and sickle cell anemia treated with the cytidine analogs, although progress in non-malignant diseases has been hampered by the potential risk of carcinogenicity that these drugs carry [22•,25•].

Clinical experience in solid tumors

5-Azacytidine

Clinical trials with 5-azacytidine are summarized in Table 1. An initial phase I trial was reported in 1972 by Weiss *et al* [26] using doses of 0.55 to 2.4 mg/kg/day for 10 to 15 days. Thirteen of thirty treated patients were reported to have had a partial response (two of six colon cancers, seven of eleven breast cancers and two of five melanomas amongst them). Remissions commonly occurred early in the treatment course and lasted an average of 6 weeks. Re-induction of remissions was possible in two patients with breast cancer who had relapses at 8 to 10 weeks. A maintenance regimen using 2.4 mg/kg twice a week was given to responding patients and two of these remained in remission for at least 6 months.

Following these encouraging results, several phase II trials were conducted. At the Mayo Clinic, 29 patients with advanced gastrointestinal cancer were treated with 500 to 750 mg/m^2 per course [27]. Nausea and vomiting were so severe, however, that three different administration schedules were tried in an attempt to decrease the symptoms. The drug was given once-daily for 5 days, once-daily for 10 days and twice-daily for 5 days. The latter was the better-tolerated regimen but was still seriously emetogenic. Only one partial response lasting 5 weeks was observed. A cooperative phase II study in 31 patients with breast cancer using 60 mg/m^2/day of intravenous 5-azacytidine for 10 days (followed by a maintenance dose of 100 mg/m^2 twice weekly after bone marrow recovery) [28] yielded only four disease stabilizations and two partial responses lasting a mean of 5.5 months. Another small phase II trial was reported in 1982 [29], where eight patients with osteogenic sarcomas and seven with skeletal Ewing's sarcoma were treated with 150 to 200 mg/m^2/day every 8 h for 5 days; no objective responses were seen.

Several large studies involving a spectrum of solid tumors have been published. The Southwest Oncology Group treated 191 patients with 5-azacytidine intravenously (225 mg/m^2/day) for 5 days every 3 weeks [30]. Because of myelosuppression, however, this dose had to be reduced initially to 175 and then to 150 mg/m^2. Two patients with adenocarcinoma of the lung, one with squamous cell carcinoma of the lung and two with embryonal carcinoma of the testicle had partial responses lasting from 28 to 77 days. Five renal, one breast, two colon and two pancreatic adenocarcinomas plus single malignancies arising from six other primary sites, were stabilized for 39 to 255 days. 6% Of patients died and 11% refused further therapy because of the drug's severe gastrointestinal toxicity. The Central Oncology Group administered 5-azacytidine (1.6 mg/kg/day) for 10 days to 221 patients [31]. In an attempt to decrease the uncontrollable nausea and vomiting induced on the day of treatment, 29 patients received the drug as an 18 to 24 h infusion. The degrees of leukopenia and thrombocytopenia were greater with the slow infusion. Stomatitis as well as an erythematous rash appeared, but nausea and vomiting, were minimal. Although 19 partial responses were reported (one lung, six breast, three lymphoma and nine miscellaneous tumors), they were mainly of non-visceral disease and short-lived (mean of 5 weeks). The Southeastern Cancer Study Group [32] tried a biweekly regimen of 150 mg/m^2 (50 mg/m^2 in lymphoma patients) of 5-azacytidine in 91 patients with disseminated malignancies, but only obtained two partial responses (one large cell carcinoma of the lung and one melanoma). In all of these trials, all of the patients that received the drug as an intravenous bolus suffered severe nausea and vomiting that was unresponsive to antiemetics, and frequently accompanied by diarrhea. Leukopenia and thrombocytopenia were dose-related and occurred late in the course of treatment.

A small study comparing intravenous and subcutaneous administration of 5-azacytidine in humans had shown that plasma levels were similar after 1 h and that the drug tended to concentrate in tumor tissue regardless of the route used [33]. Based on these results, another phase I trial was conducted using 275 to 850 mg/m^2 administered subcutaneously daily for 10 days followed by 35 to 90 mg/m^2 weekly in those who responded [34]. 18 Patients were evaluable for toxicity. Nausea, vomiting and diarrhea were mild in this trial, however, severe hepatic toxicity occurred in five patients (all with significant hepatic metastatic disease), of which three died in hepatic coma. The platelet counts of three patients dropped to < 50,000/mm^3, and two died as a direct consequence. Only two partial responses were observed which lasted 2 and 3 months, respectively.

One study investigated the benefits of administering 5-azacytidine with pyrazofurin (PF; an inhibitor of the enzyme orotidylate decarboxylase in the *de novo* pyrimidine biosynthesis pathway) after significant synergism of these drugs had been demonstrated in cell cultures [35]. 5-Azacytidine was given as a continuous intravenous infusion

Table 1. Clinical trials of 5-azacytidine in solid tumors.

Study	Number of patients (Evaluable)	Dose	Schedule	Objective responses	Reference
Phase I: Breast, colon, melanoma, lung, soft tissue sarcomas, ovary, pancreas, lymphoma	30	0.55 to 2.4 mg/kg iv bolus	qd x 10 to 15 days	13 PR	[26]
Phase II: Colon, one pancreas and one gastric	29	500 to 750 mg/m^2 iv bolus (total dose per course)	qd x 5 days; qd x 10 days; bid x 5 days	1 PR	[27]
Phase I: Ovary, breast, tonsil, lung, hepatoma, renal, colon, melanoma, chordoma	18	275 to 850 mg/m^2 sc (M: 35 to 90 mg/m^2)	qd x 10 days (M: once a week)	2 PR	[34]
Phase II: Breast	27	60 mg/m^2/day iv bolus (M: 100 mg/m^2)	qd x 10 days (M: biweekly)	2 PR 4 SD	[28]
Phase II: Solid tumors[1]	167	150 to 225 mg/m^2 iv bolus	qd x 5 days every 3 weeks	5 PR 16 SD	[30]
Phase II: Solid tumors[2]	177	1.6 mg/kg iv bolus/18 to 24-h infusion (M: 2.4 mg/kg)	qd x 10 days (M: biweekly)	19 PR	[31]
Phase II: Solid tumors[3]	91	150 mg/m^2 iv bolus	Biweekly x 6 days	2 PR	[32]
Phase I: In combination with pyrazofurin	6	30 to 60 mg/m^2 continuous iv infusion	qd x 5 days	no responses	[35]
Phase II: Sarcomas of the bone	14	150 mg/m^2 iv over 3 h	Every 8 h x 5 days	no responses	[29]

M maintenance, **qd** once daily, **PR** partial response, **SD** stable disease.
[1]Tumor and number of patients shown in parentheses: Pancreas (adenocarcinoma (6) and islet cell carcinoma (1)); lung (adenocarcinoma (12), squamous cell carcinoma (14) and undifferentiated cell carcinoma (12)); colorectal (15); kidney (17); breast (14); testicle (embryonal cell carcinoma (2), teratocarcinoma (1), choriocarcinoma (1)); urinary bladder (2); primary liver carcinoma (4); palate adenocarcinoma (1); parotid adenocarcinoma (3); uterus adenocarcinoma (1); cervix squamous cell carcinoma (4); ovary (5); non-Hodgkin's lymphoma (4); sarcoma (7); melanoma (13); tongue squamous cell carcinoma (2); nasal antrum (1); esophagus squamous cell carcinoma (3); stomach adenocarcinoma (7); skin squamous cell carcinoma (1); larynx squamous cell carcinoma (1); gall bladder (1); unknown primary (adenocarcinoma (8) and undifferentiated cell carcinoma (4)).
[2]Tumor and number of patients shown in parentheses: Lung (24); breast (29); large intestine (26); melanoma (12); Hodgkin's disease (6); non-Hodgkin's lymphoma (8); miscellaneous (59).
[3]Tumor and number of patients shown in parentheses: Breast (6); renal (10); other urogenital (3); colon (7); pancreas (2); stomach (3); rectum (1); lung (unspecified (2), squamous cell (6), adenocarcinoma (11), undifferentiated (5), small cell (2), large cell (2)); melanoma (10); head and neck (14); soft tissue sarcomas (3); lymphomas (4).

for 5 days immediately following the injection of PF. PF doses ranged from 50 to 100 mg/m^2 and 5-azacytidine doses ranged from 30 to 60 mg/m^2. The most common side effect was skin rash, which was dose-related, and when severe was accompanied by stomatitis, proctitis and cystitis. Six patients with solid tumors were entered into the trial but no objective responses were observed.

5-Aza-2'-deoxycytidine

Clinical trials with 5-aza-2'-deoxycytidine in solid tumors are summarized in Table 2. The first phase I trial conducted with 5-aza-2'-deoxycytidine used a schedule consisting of three consecutive 1-h infusions separated by 7 h [36], a schedule dictated by the instability of 5-aza-2'-deoxycytidine in aqueous solution and its short half-life. The starting dose was 25 mg/m^2. The dose-limiting toxicity consisted of reversible myelosuppression, with the white blood cell count nadir delayed to days 22 to 33 of treatment. Platelet nadir was observed between days 14 and 22. The maximum tolerated dose was 100 mg/m^2 x 3. One partial response was observed in a patient with a locally recurrent undifferentiated carcinoma of the ethmoid sinus who continued 5-aza-2'-deoxycytidine treatment every 5 to 6 weeks and, after surgery of a residual lymph node metastatasis, remained free of disease at 15 months. Based on these results, the EORTC conducted phase II trials with 5-aza-2'-deoxycytidine in patients with melanoma and colorectal, renal and head and neck cancers, using the same schedule evaluated in the phase I study, at a dose of 75 mg/m^2 [37]. Of 82 evaluable patients, only one short-lived partial response was observed in a patient with malignant melanoma. Tumor stabilization was reported in 22% of the patients with melanoma, in 15% of the patients with head and neck cancer, in 14% of the patients with renal cell carcinoma and in 7% of the patients with colorectal carcinoma. Despite the lack of significant activity in these studies, the EORTC used the same schedule and dose in 14 patients with non-seminomatous testicular cancer, because of the postulated activity of 5-aza-2'-deoxycytidine as a differentiating agent, but no objective responses were seen [38]. The same dose and schedule were also used in three other phase II trials in patients with uterine cervical cancer (n = 14), ovarian cancer (n = 21) and prostate cancer (n = 12). No responses were seen in the uterine cervical cancer group but stabilization of disease was observed in two patients with ovarian cancer and two patients with prostate cancer [39-41]. The most common non-hematologic toxicity encountered in all trials with this regimen was mild-to-moderate nausea and vomiting.

Table 2. Clinical trials of 5-aza-2'-deoxycytidine in solid tumors.

Study	Number of patients (Evaluable)	Dose	Schedule	Objective responses	Reference
Phase I	20	25 to 100 mg/m^2 over 1 h every 8 h x 2 to 3	Every 3 to 6 weeks	1 PR	[36]
Phase II: Melanoma, colorectal, renal and squamous cell cancer of the head and neck	82	75 mg/m^2 over 1 h every 8 h x 3	Every 5 weeks	1 PR and 13 SD	[37]
Phase II: Ovarian cancer	21	75 mg/m^2 over 1 h every 8 h x 3	Every 5 weeks	2 SD	[40]
Phase II: Cancer of the uterine cervix	14	75 mg/m^2 over 1 h every 8 h x 3	Every 5 weeks	no responses	[39]
Phase II: Non-seminomatous testicular cancer	14	75 mg/m^2 over 1 h every 8 h x 3	Every 5 weeks	no responses	[38]
Phase I/II: Non-small cell lung cancer	9	200 to 660 mg/m^2 x 1 over 8 h	Every 5 to 7 weeks	4 SD	[43]
Phase II: Prostate cancer	12	75 mg/m^2 over 1 h every 8 h x 3	Every 5 to 8 weeks	2 SD	[41]
Phase I	21	45 to 120 mg/m^2 over 1 h + 33 mg/m^2 cisplatin every 24 h x 3	Every 3 weeks	1 PR and 2 minor responses	[47]
Phase II: Non-small cell lung cancer	14	67 mg/m^2 over 1 h + 33 mg/m^2 cisplatin every 24 h x 3	Every 3 weeks	3 minor responses	[47]
Phase I	19	20 to 40 mg/m^2/day over 72 h	Every 5 weeks	no responses	[44]

Both *in vitro* and *in vivo* data [42] suggested that the cytotoxicity of 5-aza-2'-deoxycytidine was dose- and time-dependent. Therefore, alternative schedules of administration were explored. In 1997 Momparler *et al* conducted a phase I/II trial in previously untreated patients with stage IV non-small cell lung cancer, using an 8-h intravenous infusion of 5-aza-2'-deoxycytidine at a dose of 200 to 660 mg/m^2. They reported an increase in survival time with the number of cycles administered and one long-term survivor. They concluded that 5-aza-2'-deoxycytidine has a delayed action on tumor growth that may require several cycles of treatment before becoming evident [43]. Our group conducted a phase I trial using a 72-h continuous intravenous infusion of doses between 20 and 40 mg/m^2/day. All of the patients were heavily pretreated and only one of the 19 was able to receive more than one cycle of treatment. No objective tumor responses were observed [44].

In vitro studies have demonstrated synergistic cytotoxicity using 5-aza-2'-deoxycytidine and cisplatin, 4-hydroperoxycyclophosphamide (a derivative of cyclophosphamide) and topotecan [45,46]. Based on these results, a phase I trial was conducted by Schwartsmann *et al* [47] using cisplatin (33 mg/m^2) plus 5-aza-2'-deoxycytidine (45 to 120 mg/m^2) as a 2-h infusion on days 1 to 3. One partial response was observed in a patient with advanced cervical cancer. A follow-up early phase II evaluation in 14 patients with inoperable non-small cell lung cancer used 5-aza-2'-deoxycytidine (67 mg/m^2) and cisplatin (33 mg/m^2) on days 1 to 3. Only three short-lasting minor regressions were observed [47].

Detection of DNA methylation

To clinically assess the utility of alterations in DNA methylation, it is important to accurately measure the changes in methylation that occur after treatment with demethylating agents. Until recently, the techniques available for this have been difficult to apply to clinical samples.

Initial protocols to detect changes in DNA methylation employed digestion of genomic DNA with methylation-sensitive restriction enzymes (characterized by their inability to cleave sequences that contain methylated CpG dinucleotides) followed by Southern blot analysis [1•]. The main drawbacks to this method included a requirement for large amounts of DNA (> 5 µg) and that the extent of analysis was limited to the CpG sites present in the recognition sites of the available enzymes. Later on, PCR amplification was applied using primers that flanked the restriction sites that were methylation sensitive. Although the sensitivity of the assay increased, this method could still only be used to assess CpG methylation at methylation-sensitive restriction sites, and it had the potential of generating false positive results if the cleavage of the unmethylated DNA was incomplete [48].

Genomic sequencing was also used to detect 5-methylcytosine residues, identified as a lack of bands in all tracks of a sequencing gel. However, interpretation was frequently complicated by close spacing of the bands or by background cleavage ladders [1•]. In 1992, Frommer *et al* [49] described the bisulfite genomic sequencing technique based on treatment of single stranded DNA with sodium bisulfite to deaminate cytosine to uracil much faster than 5-methylcytosine to thymine, so that methylated cytosine residues are left intact. The bisulfite-treated DNA was amplified by PCR and the products sequenced. The 5-methylcytosine residues on the original sample appeared as the only remaining cytosines on the sequencing gel, since unmethylated cytosines were transformed to uracil by the bisulfite. This approach could be applied to small amounts of DNA; even DNA obtained from paraffin-embedded tissue samples. However, without cloning the amplified products, a labor-intensive and time-consuming process, this method was less sensitive than Southern analysis. Nevertheless, bisulfite treatment of genomic DNA provided the basis for multiple new strategies for the assessment of DNA methylation.

Herman *et al* [50] described the methylation-specific PCR protocol in which they used three sets of primers designed specifically to amplify three types of bisulfite-treated DNA: methylated, unmethylated and DNA that had not been modified. The PCR products were compared on polyacrylamide gels stained with ethidium bromide, providing semiquantitative results. This method was highly sensitive, capable of detecting even 0.1% of methylated DNA in a sample, and significantly less time consuming than genomic sequencing. Gonzalgo and Jones [48] developed the methylation-sensitive single nucleotide primer extension assay in which the PCR product from the bisulfite-converted DNA amplification was isolated from an agarose gel and used as a template for a second PCR reaction. The second PCR reaction utilized ^{32}P-labeled dNTPs and internal primers that terminated immediately 5' of the single nucleotide of interest. The radiolabeled products were electrophoresed resulting in two bands: the C band represented the methylated cytosine residues and the T band represented the unmethylated cytosine residues. Phosphorimage analysis allowed for quantitation of each band. At the same time, Xiong and Laird [51] reported a combined bisulfite restriction analysis, that involved a standard sodium bisulfite PCR treatment, followed by the digestion of the purified PCR products using a restriction enzyme with CpG in its recognition sequence. In this way, cleavage only occurred if the CpG sequence had been retained during the bisulfite conversion, ie, if the original C residue was methylated. Different restriction enzymes detected different levels of DNA methylation, depending on the number of CpG dinucleotides contained in its recognition sequence. Gel electrophoresis, oligo hybridization and phosphorimage analysis allowed quantitation of the level of methylation present. Although these methods were rapid, offered quantitative results and were compatible with paraffin embedded tissues, they still required cumbersome manipulations and were not easily applicable to large numbers of specimens.

Finally, Eads *et al* [52] developed a high-throughput quantitative assay for the analysis of DNA methylation, called MethyLight. This technique begins with sodium bisulfite conversion of the sample DNA, which is then amplified by PCR using three methylation-specific oligonucleotides: a probe with a 5'-fluorescent reporter dye and a 3'-quencher dye, and two locus-specific PCR primers that flank the probe. During the PCR reaction, the nuclease activity of the Taq DNA polymerase cleaves the probe and the reporter is released resulting in a fluorescent signal that is proportional to the amount of PCR product generated and measurable with a real-time fluorescence instrumentation. This technology has made possible the rapid screening of large numbers of human tumors for the methylation state of a particular locus [53] but cannot offer information on longer regions of DNA.

The ideal technique to analyze the biological activity of demethylating agents in clinical trials should be able to combine the high-resolution information of the methylation state of a large area of DNA sequence, like bisulfite sequencing, with the sensitivity, accuracy and efficiency of high-throughput technology.

Future directions

Since DNA methylation plays an important role in the regulation of gene expression, modulating it pharmacologically is a very attractive therapeutic target. 5-Azacytidine and 5-aza-2'-deoxycytidine have not been dramatically successful, and pose significant restrictions on dosing schedule adjustments because of their instability in aqueous solution and their side effects. Development of new drugs without these limitations is a subject of active research. Other approaches to inhibit DNMT1, the enzyme responsible for the replication of the DNA methylation pattern, are being explored, including the use of modified oligonucleotides that directly antagonize the enzyme [54]. Antisense oligonucleotides have been tested in the laboratory [55] and clinical trials have moved ahead [56•]. Combinations of demethylating agents with synergistic drugs, such as the histone deacetylase inhibitors (trichostatin A [57] and 4-phenylbutyrate [58]), or targeting specific signal transduction pathways that render cells sensitive to chemotherapy agents, are other strategies that should be further investigated.

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Characterization of acid–base properties of unstable drugs using a continuous-flow system with UV–vis spectrophotometric detection

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Abstract

In this paper, we propose a continuous-flow system for the study of the acid–base characteristics of unstable drugs. 5-Azacytidine has been selected as a first model of unstable compound, which progressively decomposes in aqueous solutions. Besides, other compounds undergoing hydrolysis and oxidation side reactions have been also analyzed to explore the performance of the method. In comparison with conventional batch titrations, the drug decomposition can be minimized by the continuous renewal of the analyte solution. The composition of the buffer mixture is varied on-line during the process from successive changes in the flow rates of acid and basic stock solutions. As a result, the pH value of the test solution is varied in a controlled manner in the range of 1–13. Multivariate curve resolution based on alternating least squares has been used to extract relevant information concerning the acid–base properties of analytes. Results from the continuous-flow system have been compared with those obtained, using batch spectrophotometric titrations, and in the case of fast degradations, the performance of the proposed procedure has been superior.

Keywords: Unstable drugs; 5-Azacytidine; Acid–base characterization; Continuous flow titration; Chemometric analysis

1. Introduction

Physicochemical properties of drugs, and especially acid–base constants, are of great importance in the pharmaceutical and clinical fields, as they influence the ADME profiles [1,2]. In the past decades, batch pH-metric and spectrophotometric titrations were considered as the reference methods for pK_a determinations [3]. Despite the passage of time, nowadays, these methods are being extensively applied to the calculation of dissociation constants. Some relevant features are the excellent precision and accuracy although certain solubility and stability problems may arise. Recent trends in determination of dissociation constants are based on predictive models established from correlations of chemical parameters of organic compounds [4,5], and chromatographic [6–9] and electrophoretic methods [10–17]. The use of separation techniques for pK_a determinations is based on the variation of the retention time or electrophoretic mobility as a function of pH. Apart for experimental methods, various computer programs such as PALLAS [18] and SPARC [19] have been developed for the estimation of dissociation constants from the chemical structure of the test compounds.

The lack of stability of the test solution during the measurement period may result in a significant drawback common to most of the experimental methods. In the case of unstable compounds, side reactions such as hydrolysis, oxidation, and precipitation may occur in parallel to the titration procedure so that the quality of the results may be affected. Flow methods based on a continuous renewal of the analyte solution during the experimental study have been proposed for solving this shortcoming. Besides, flow methods may offer additional advantages such as rapid analysis, excellent reproducibility, high degree of automation, and low expense of reagents [20,21]. Box et al. have proposed a flow system for the generation of a linear pH-gradient as a function of time from the mixture of acid and basic stock buffer solutions [22]. A computer-controlled pumping device is used to deliver solutions while providing the appropriate buffer composition. This system has been commercialized as the SGA-Profiler from Sirius for rapid determination of pK_a values of drugs with a sample throughput of 4 min per

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assay (see www.sirius-analytical.com). Other papers describe the application of a pH-gradient flow-injection system for the estimation of acidity constants in which the whole titration is carried out in a few seconds. In this case, the pH gradient profile is first calibrated using suitable standards, and in a second stage, this pH profile is applied to calculate the pK_a values of test drugs [23,24].

The treatment of pH-metric and spectrophotometric data obtained in the acid–base studies has been carried out traditionally with computational methods such as SQUAD or SUPERQUAD as described in the Leggett's book [25]. These methods have not fallen into disuse but they have been updated with novel non-linear approaches for more stable and efficient modelling [26,27]. In last years, other treatments based on factor analysis have been proposed. Among them, target factor analysis (TFA) has been used extensively for the determination of acidity constants of organic compounds [28–31]. TFA estimates the abstract spectral and pH profiles from principal component analysis (PCA) and applies further rotation of factors to get meaningful contributions of the chemical species. TFA results in a simple chemometric method, which does not require special training, and can be easily automated. Another factor analysis method called multivariate curve resolution, based on alternating least squares (MRC-ALS) was proposed by Tauler et al. at the beginning of the nineties for the characterization of acid–base processes [32]. Since then, the method has been continuously improved with the implementation of natural constraints and model conditions for enhancing the analytical performance [33]. The simultaneous analysis of various processes, that is, various experimental data sets, has offered new possibilities for solving resolution ambiguities, and improving the precision and accuracy [34].

In this paper, 5-azacytidine has been selected as a model compound of unstable drugs. 5-Azacytidine is used as an anticancer agent belonging to nucleoside analogs [35–37]. The scheme of the deprotonation reactions is given in Fig. 1(a). Regarding stability, this drug decomposes progressively in aqueous solutions according to the reaction shown in Fig. 1(b) [38,39], this degradation being especially dramatic in basic media. For this reason, batch titrations are not suitable for evaluating the acidity constants due to its parallel hydrolysis in the titration vessel during the experimental procedure. Apart form 5-azacytidine, other unstable drugs undergoing hydrolysis, oxidation, or other side reactions, have been analyzed in this paper to check the performance of the proposed method in different circumstances.

The flow procedure proposed here consists of a continuous mixing of test solution with the titrant buffer solution using a two-pump manifold. The method has been adapted from a previous publication by Saurina et al. [40], which developed, and evaluated a continuous flow titration system for the spectrophotometric characterization of acid–base reactions. A similar approach was used in the study of the derivatization of amino acids with 1,2-naphthoquinone-4-sulfonate as a function of pH [41]. In these systems, the time of contact between analyte and buffer was short enough to avoid any decomposition, even at basic pH values. During the experimental procedure, the composition of the buffer solution (and thus the pH), was sequentially varied on-line from varying the ratio of the acid and basic stock solutions. Data generated in each run consisted of spectra registered at different pH values in the range of 1 to 13, approximately. MCR-ALS was used for data analysis in order to recover the acid–base profiles of species, and to calculate the corresponding acidity constants [42,43].

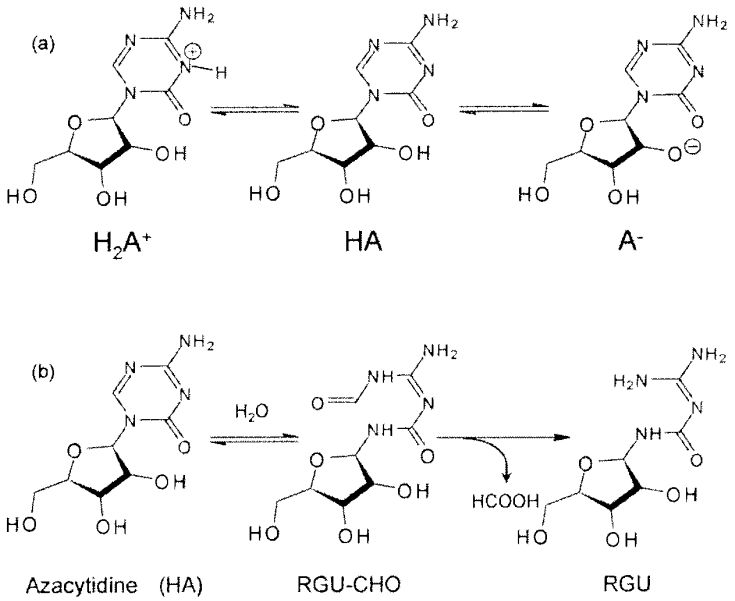

Fig. 1. Scheme of the protonation (a) and decomposition reactions (b) of 5-azacytidine. Species assignment: H_2A^+, protonated 5-azacytidine species; HA, neutral 5-azacytidine species; A^-, deprotonated 5-azacytidine species; RGU-CHO, N-(formylamidino)-*N'*-ß-D-ribofuranosylurea; RGU, 1-ß-D-ribofuranosyl-3-guanylurea.

2. Materials and methods

2.1. Reagents and solutions

Ultrapure water (Millipore, Milford, MA, USA) was used for the preparation of all solutions. Test compounds were 5-azacytidine, dopamine, didanosine, pyrocatechol, sodium naphthoquinone-4-sulfonate, thioguanine, tyramine, and tyrosine from Sigma-Aldrich (St. Louis, MO, USA), and triflusal (2-acetoxy-4-(trifluoromethyl) benzoic acid) from Uriach (Barcelona, Spain). Sodium acetate, boric acid potassium dihidrogenfostate, hydrochloric acid, and sodium hydroxide (all of them from Merck, Darmstadt, Germany), were used for the preparation of acid and basic stock solutions indicated below. Standard buffer solutions of pH 7.0 and 4.0 for the calibration of the glass electrode were purchased from Panreac (Barcelona, Spain).

The acid stock solution to be used in the flow system consisted of 0.05 M phosphoric acid + 0.05 M acetic acid + 0.05 M boric acid. The basic stock buffering solution was composed of 0.05 M phosphate + 0.05 M borate + 0.05 M acetate.

2.2. Apparatus

A Perkin-Elmer Lambda-19 spectrophotometer equipped with a Helma flow-cell of 10 mm path length and 60 μl volume was used for spectral measurements in the range of 220–300 nm. Spectroscopic data were acquired with a PC using the standard Perkin-Elmer software. The pH of the sample solution was measured in the waste solution emerging from the system with a CyberScan model 2500 pHmeter (precision of ±0.1 mV) using a combined pH electrode ORION 9103SC with an inner Ag/AgCl reference electrode.

2.3. Continuous flow procedure

The two-pump experimental set-up was adapted from the former system proposed by Saurina et al. for the study of acid–base reactions [40]. As shown in Fig. 2, the continuous flow manifold was composed of three channels for pumping sample, acid and basic stock solutions, and two peristaltic pumps (Watson Marlow 505DU) P1 and P2. The buffer (titrant) solution was obtained by mixing on-line these acid and basic stock solutions in a PTFE mixing coil (200 cm × 0.7 mm I.D.). During the procedure, the speed of P2 was varied sequentially which lead to a variation in the buffer composition, and thus, in the pH of the resulting solution. Subsequently, sample and buffer solution mixed in a PTFE reaction coil (35 cm × 1.1 mm I.D.), reached the detection flow cell in 9 s. After each modification of the P2 speed, the corresponding spectrum was registered under steady-state conditions and the pH was measured experimentally at the waste solution.

In more detail, the peristaltic pump P1 delivered sample and buffer solutions at a constant flow rate of 1.15 ml min^{-1} each channel. In the titration, the flow rate of the acid stock solution was varied from 1.9 to 0.0 ml min^{-1}, using the variable-speed pump P2. In parallel, the basic stock solution was aspired at a variable flow rate (from 0.4 to 2.3 ml min^{-1}), which resulted

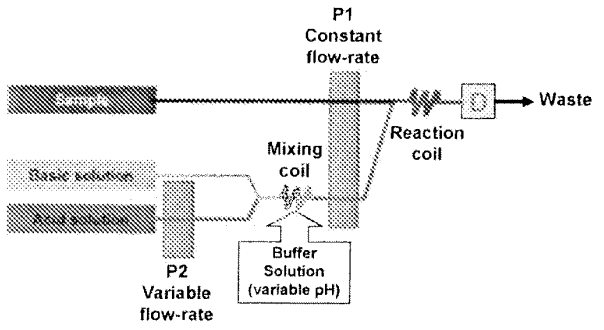

Fig. 2. Scheme of the continuous-flow system for the characterization of acid–base properties of unstable compounds. P1, Constant-speed peristaltic pump; P2, variable-speed peristaltic pump; D, spectrophotometer (spectral range: 220–300 nm); mixing coil = 200 cm × 0.7 mm I.D.; reaction coil = 35 cm × 1.1 mm I.D.; sample = 5 × 10^{-5} M 5-azacytidine solution; acid (stock) solution = 0.05 M phosphoric acid + 0.05 M acetic acid + 0.05 M boric acid; basic (stock) solution = 0.05 M phosphate + 0.05 M borate + 0.05 M acetate. Flow rates: sample channel = 1.15 ml min^{-1}; buffer channel = 1.15 ml min^{-1}; acid channel, variable from 1.9 to 0.0 ml min^{-1}; basic channel, variable from 0.4 to 2.3 ml min^{-1}.

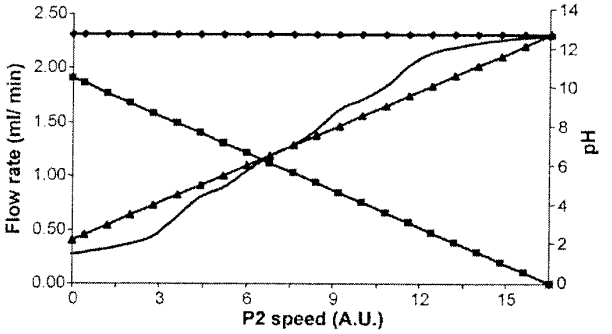

Fig. 3. Variation of the pH of the buffer solution as a function of the variation of the flow rates of acid and basic channels. –■–, Acid channel flow rate; solution; –▲–, basic channel flow rate; –♦–, titrant channel flow rate; —, pH profile.

from the difference between the flow rates of buffer and acid channels. Fig. 3 shows the variation of the flow rate of acid and basic stock solutions as a function of the speed of P2 (in arbitrary units). Complementarily, the corresponding variation of pH is also given.

3. Chemometric treatment

The spectroscopic data generated in each acid–base run were arranged in a data matrix **D**, in which each row represented a pH value, and each column a wavelength. Hence, the elements of the matrix consisted of absorbance values as a function of pH (through the pH domain) and wavelength (spectral domain). The experimental data were further analyzed with multivariate curve resolution based on alternating least squares (MCR-ALS method) to recover the concentration, **C** (i.e., distribution of species), and spectral profiles, $\mathbf{S}^T$, of species as shown in Fig. 4.

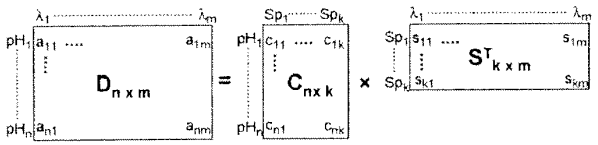

Fig. 4. Scheme of the resolution of the spectroscopic data matrix **D** into to the acid–base distribution profiles **C** and the spectral profiles $\mathbf{S}^T$ of species. Sp_i, acid–base species; a_{ij}, absorbance value at pH i and wavelength j; c_{ik}, concentration value of species k at pH i; s_{kj}, absorptivity of species k at wavelength j.

The principal steps of MCR-ALS are schematized in Fig. 5. For a more extensive description, see references [40–43]. Here, only a brief explanation of the procedure is given as follows.

3.1. Exploratory analysis

The first step consisted of a preliminary inspection of the corresponding data set using exploratory factor analysis tools such as singular value decomposition (SVD), PCA, and evolving factor analysis (EFA). This study provided relevant information concerning the number of chemical species present in the system as well as a first approximation to the analyte contribution profiles.

3.2. Initial estimations

They were the guesses of the spectral or pH profiles of species, that is, $\mathbf{S}^T$ or **C**, to be used in the optimization step. In this paper, spectral estimations were taken at the most representative pH values according to the predominance of 5-azacytidine species H_2A^+, HA, and A^-. Analogous considerations were followed for the other compounds under study.

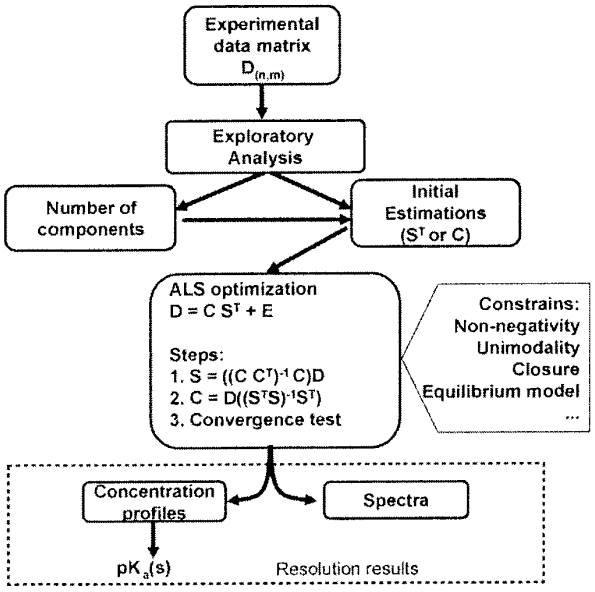

Fig. 5. Flow chart of the multivariate curve resolution method based on alternating least squares.

3.3. Optimization procedure

The least square optimization based on the compliance of the Beer's Law decomposed the experimental data matrix **D** into the distribution (concentration) profiles as a function of pH **C** and spectra $\mathbf{S}^T$. The general model can be written as: $\mathbf{D} = \mathbf{C} \times \mathbf{S}^T$.

At this stage, the initial spectral estimates were used as an input in the iterative optimization for the calculation of the concentration contributions as follows: $\mathbf{C} = \mathbf{D} \times (\mathbf{S}^T)^+$, where the superindex + refers to the generalized inverse.

Subsequently, $\mathbf{S}^T$ was updated using the expression: $\mathbf{S}^T = (\mathbf{C})^+ \times \mathbf{D}$, where $(\mathbf{C})^+$ is the generalized inverse of **C**.

In order to get better resolution results, various constrains were applied to restrict the mathematical solutions: non-negativity in the spectral and concentration profiles, unimodality, closure in the concentration profiles, and acid–base model.

The iterative calculations were repeated until obtaining the optimum **C** and $\mathbf{S}^T$ profiles. The stopping criteria were: (i) reaching a convergence fitting error defined beforehand, (ii) exciding a predefined number of iterations, and (iii) diverging in the fitting process 20 times consecutively.

The pK_a values of the test compounds were determined from the concentration profiles **C** at those points in which the concentrations of the acid and conjugate base were equal.

4. Results and discussion

4.1. Study of azacytidine processes

4.1.1. Optimization of the flow manifold

The rapid decomposition of 5-azacytidine in basic media was first evidenced in the study of spectroscopic data obtained from conventional acid–base titrations. The presence of a significant percentage of degradation occurring in parallel to the titration was demonstrated using chemometric tools. In particular, the SVD analysis showed the presence of, at least, five chemical species. Hence, apart from the three species involved in the deprotonation reactions, two additional components associated with degradation reactions were detected. This finding indicated that batch titrations were not suitable for the characterization of acid–base features of 5-azacytidine. In these circumstances, the performance of the continuous-flow assembly described in the experimental section was investigated as a way to overcome parallel degradation processes. Note that, 5-azacytidine was basified on-line, and the time of contact between the test drug and the buffer solution could be adjusted to a desired value in order to avoid decomposition.

Optimization studies were focused on the design of a set-up to minimize the drug degradation. As commented, the key aspect in this optimisation, was decreasing the residence time, which depended on both reaction coil dimensions and flow rate. The effect of the reaction coil dimensions on the 5-azacytidine decomposition was evaluated using a constant flow-rate of 1.3 ml min^{-1} through the reactor. Various reactors of different lengths and internal diameters were checked. Table 1 summarizes the results obtained, suggesting that the residence

Table 1
Study of the degradation of the 5-azacitidine underwent in the continuous-flow manifold as a function of the residence time

Optimized variable	P1 flow rate (ml/min)	Reaction coil dimensions (cm × mm I.D.)	Residence time (s)	Degradation
Reaction coil	1.3	550 × 0.7	98	Very high
		95 × 0.8	22	Slightly
		35 × 1.1	15	Almost negligible
Flow rate	0.5	35 × 1.1	37	High
	1.0		20	Slightly
	1.5		13	Almost negligible
	2.3[a]		9	None[a]
	2.7[b]		7	None[b]

[a] Selected flow rate.
[b] High noise and bubble formation.

time should be, at least, shorter than 15 s to avoid degradation.

The influence of the flow-rate on the decomposition was studied using a reaction coil of 35 cm length × 1.1 mm I.D. Flow rates of sample and buffer channels were equal, and the overall flow-rate was varied from 0.5 to 2.7 ml min^{-1}. Accordingly, residence times varied from 37 to 7 s, approximately. As indicated in Table 1, the degradation at long residence times was noticeable, while it was almost negligible for times shorter than 10 s. However, it should be mentioned that the use of high flow-rates produced undesired effect such as an increase in the level of noise and risk of formation of bubbles. The optimum conditions corresponded to an overall flow-rate of 2.3 ml min^{-1} and a 35 cm length × 1.1 mm I.D.

4.1.2. Resolution of 5-azacytidine spectroscopic data

As an example, Fig. 6(a) shows the spectroscopic data obtained in the range of pH from 1 to 13 for the analysis of a 5×10^{-5} M 5-azacytidine solution, using the proposed flow-method. Spectral changes were noticeable around pH 3 and 11, approximately, so the corresponding pK_a(s) should be close to these values. As shown in the protonation scheme of Fig. 1(a), the first deprotonation of 5-azacytidine (H_2A^+) occurred in N-3 of the triazin-2-one ring. The pK_2 was associated to the deprotonation of the hydroxyl group in position three of the ribofuranosyl ring.

The MCR-ALS method was applied to the analysis of the spectroscopic data for the resolution of spectra and distribution profiles of drug species. The SVD analysis indicated the presence of three significant chemical species and no evidence of further degradation. Spectral initial estimations of H_2A^+, HA, and A^- were chosen at pH 1, 7, and 13 as they predominate around these pH values.

The ALS algorithm was applied to extract the 5-azacytidine profiles. The following constrains were used in order to improve the quality of the resolution: (i) Unimodality in the concentration profiles, (ii) non-negativity in both spectral and concentration profiles, (iii) physicochemical model based on the compliance of the mass-action law, (iv) closure in the concentration profiles.

Fig. 6 shows the results recovered from the MCR-ALS analysis. In the case of the concentration profiles (Fig. 6(b)), the crossing points corresponded to the pK_a values of 5-azacytidine. Spectra of H_2A^+ and HA species were quite similar (with peak maxima around 255 and 245 nm, respectively), while A^- species displayed higher molar absorptivity.

This type of experimental studies was carried out in triplicate, so that analogous data were obtained from the other runs. As summarized in Table 2, results from the MCR-ALS were similar in the three analyses. The average pK_a values of 5-azacytidine were 2.67 ± 0.03 and 12.26 ± 0.02.

4.2. Study of other unstable drugs

Additional examples of other unstable compounds were analyzed according to the proposed method, in order to evaluate its performance for different model compounds. In particular, the following compounds were selected: Triflusal (2-acetoxy-4-(trifluoromethyl) benzoic acid) as a model of ester compound related to acetylsalicylic acid, which is being commercialized as a platelet aggregation inhibitor. The ester group undergoes a rapid hydrolysis in basic media. Didanosine and thioguanine were chosen as antiretroviral and anticancer nucleoside analogues suffering hydrolysis reactions in basic media. Apart from hydrolysis reactions, other compounds such as tyrosine, tyramine, and dopamine were selected as examples of oxidizable phenolic derivatives. More complex side reactions were expected in the case of pyrocatechol, which was analyzed as an example of polyphenol compound undergoing oxidation and condensation reactions. Finally, naphthoquinone-4-sulfonic acid was chosen as a model of substances with oxidation and hydrolysis problems.

These compounds were studied in a similar manner to 5-azacytidine case detailed in the previous section. As a com-

Table 2
Values of pK_a of 5-azacitidine calculated using the proposed procedure

Titrations	Experimental pH range	pK_{a_1}	pK_{a_2}
T1	1.40–12.65	2.66	12.25
T2	1.40–12.61	2.70	12.25
T3	1.63–12.54	2.64	12.29
Average		2.67 ± 0.03	12.26 ± 0.02

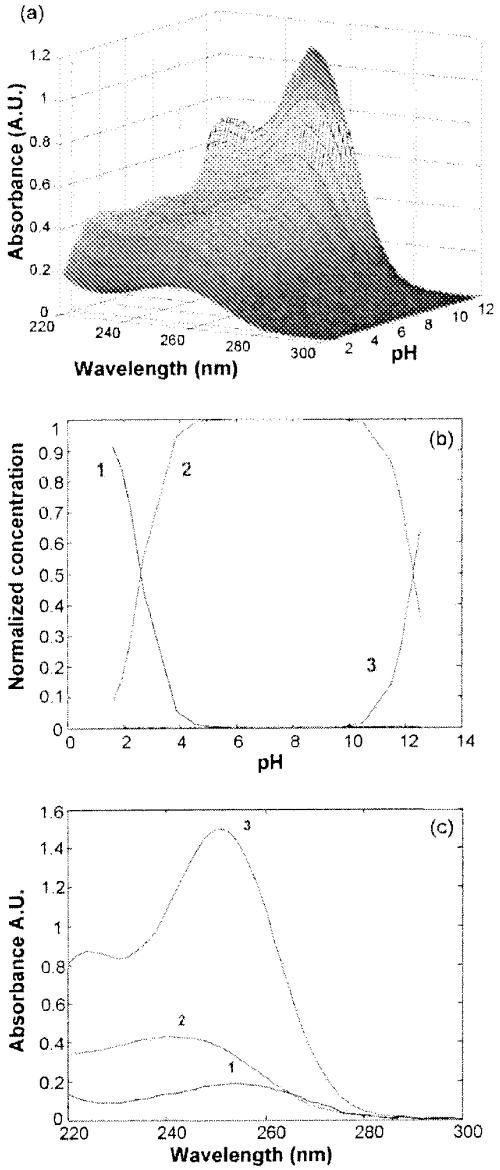

Fig. 6. Determination of the pK_a values of 5-azacytidine. (a) Spectroscopic data obtained for a 5×10^{-5} M 5-azacytidine; (b) concentration (distribution) profiles recovered using the MCR-ALS and (c) spectral profiles recovered using the MCR-ALS. Species assignment: 1, H_2A^+; 2, HA; 3, A^-, defined according to Fig. 1a.

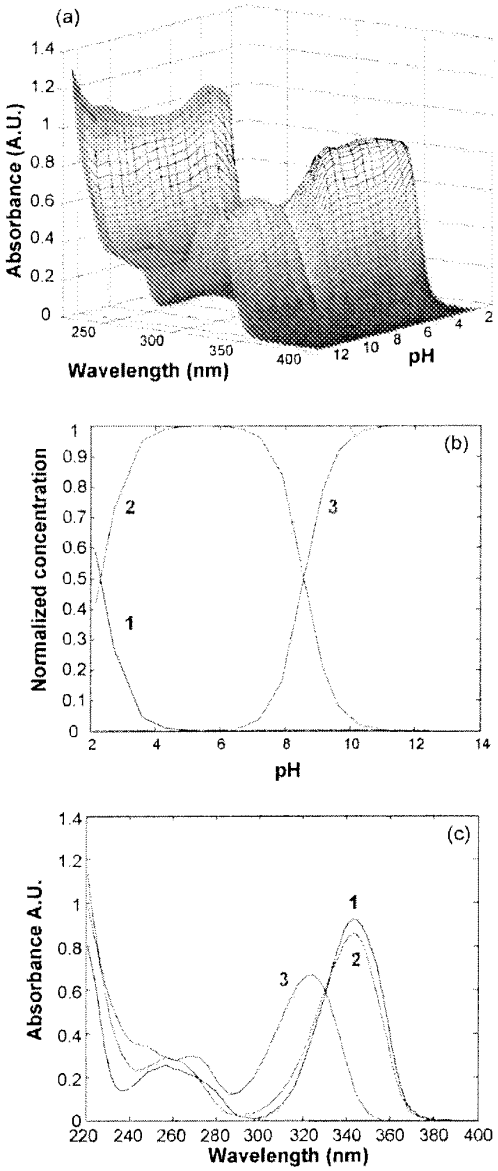

Fig. 7. Determination of the pK_a values of thioguanine. (a) Spectroscopic data obtained for a 5×10^{-5} M thioguanine; (b) concentration (distribution) profiles recovered using the MCR-ALS and (c) spectral profiles recovered using the MCR-ALS.

plementary example, the characterization of thioguanine has been illustrated with figures depicting the experimental data set as well as the resolved spectral and distribution profiles (see Fig. 7).

The pK_a values calculated according to the proposed procedure were compared with those available in the scientific literature (see Table 3). As indicated, in the case of didanosine and thioguanine, dissociation constants were obtained from a pH-gradient flow-injection method, which was able to minimize extension of degradation processes. In the absence of suitable published values as a reference, pK_a values were estimated from the calculation programs (PALLAS and SPARC) based on the structure of the molecules. The lack of some experimental values was attributed to the existence of degradation processes, which may hinder the calculation of pK_a values. As shown in Table 3, a satisfactory concordance was found in all cases so that the accuracy and efficiency of the proposed procedure was demonstrated.

Table 3
Determination of pK_a values of some model compounds using the proposed method

Compound	Proposed method	Reference method		
	Experimental pK_a	Reference pK_a	Comments	Reference
Didanosine	9.50	9.35	pH-gradient FIA	[24]
Dopamine	8.73	8.87	Potentiometry	[44]
1,2-Naphthoquinone-4-sulfonic acid	9.08	8.86	PALLAS estimation	[18]
	10.90	11.68		
Pyrocatechol	9.56	9.45	Potentiometry	[3]
6-Thioguanina	2.30	–	pH-gradient FIA	[24]
	8.45	8.25		
		2.30	SPARC estimation	[19]
		8.60		
Triflusal	2.97	3.04	PALLAS estimation	[18]
Tyramine	9.58	9.53	Potentiometry	[44]
Tyrosine	9.24	9.21	Potentiometry	[3]

5. Conclusions

The continuous flow method resulted in a highly attractive and efficient approach for a rapid characterization of acid–base properties of drugs. The performance of this method was demonstrated in the case of unstable compounds in which batch procedures may fail. The time of contact between analyte and buffer solution was modulated from the reaction coil dimensions and total flow-rate to minimize the degradation process. For 5-azacytidine, the degradation in basic media was noticeable in less than 1 min, so that shorter residence times were required. The method was used with no modification to the analysis of other unstable compounds such as didanosine, naphthoquinones sulfonate, etc. The system could be even adapted for dealing with faster degradations through re-optimization of the manifold variables for getting shorter residence times. Multivariate curve resolution based on alternating least squares was used to recover the contributions associated to the chemical species from the treatment of the experimental data sets. The number of species detected mathematically was consistent with the acid–base reactions so that possible parallel decompositions were avoided. In comparison with commercial instruments, the system proposed here can be easily assembled from apparatus and pieces commonly present in the majority of pharmaceutical and analytical laboratories with no extra cost, and it can be used for both eventual and routine determinations. The versatility of the method can be extended through the use of other detectors of interest when test drugs display poor spectrophotometric features.

Acknowledgement

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Synthesis and Antitumor Activity of Dihydro-5-azacytidine, a Hydrolytically Stable Analogue of 5-Azacytidine[1]

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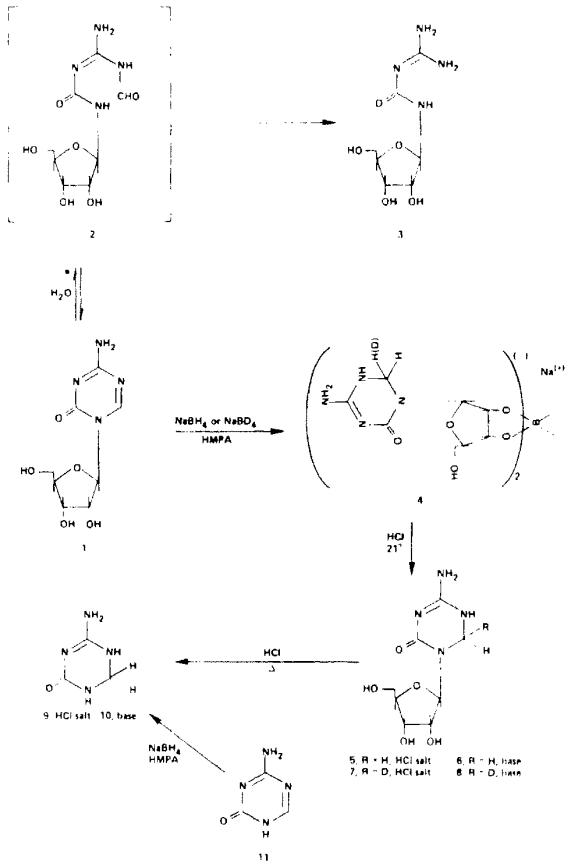
Full clinical utility of the antileukemic drug, 5-azacytidine (1), is hampered by its facile hydrolysis in aqueous formulations. The present study sought to improve the stability of the parent drug while retaining the antitumor attributes through the synthesis of a reduced analogue of 1. Borohydride reduction of 1 gave 5,6-dihydro-5-azacytidine hydrochloride (5) after acid hydrolysis of a boron-containing intermediate. The structure proof and characterization of 5 was achieved primarily with UV, NMR, and GC–MS with the aid of a deuterated derivative (7) prepared by using borodeuteride in the initial reduction step. Vigorous treatment of 5 with acid gave the aglycon 9 which was independently synthesized from 5-azacytosine (11). The dihydro analogue 5 was completely stable at room temperature in aqueous solutions over a broad pH range for up to 3 weeks. In comparative antitumor assays 5 showed good activity in L1210 systems when administered intraperitoneally or orally. Although higher dose levels were necessary, 5 had approximately 80% of the antitumor efficacy shown by 1. Neither 5 nor 1 showed a dependency on administration schedule. Cross resistance between 5 and 1 was demonstrated using an L1210 subline resistant to 1. 5 was found to be superior to 1 in therapeutic index and in its ability to cross the blood-brain barrier in sufficient quantity to be therapeutic against intracranially implanted L1210 cells. Subjective evidence is given which suggests 5 is a prodrug of 1.

5-Azacytidine (1) is a nitrogen bioisostere of cytidine conceived as a potential inhibitor of nucleic acid biosynthesis and synthesized by Piskala and Sorm[3] in 1964; later it was also isolated from the culture filtrates of *Streptoverticilium ladakanus*.[4] The nucleoside was reported to have antibacterial properties[4,5] and to be a potent inhibitor of rapidly proliferating murine neoplasms.[4] 5-Azacytidine is incorporated into both RNA and DNA[6] and disrupts protein synthesis, probably through its incorporation into messenger RNA.[7] The noteworthy activity of 1 in experimental biological systems and antitumor assays encouraged clinical trials of 1 in the treatment of leukemia.[8–10] Although of apparent limited value in the treatment of solid tumors,[11,12] 5-azacytidine has been particularly effective against acute myelogenous leukemia.[9,10]

The primary dose-limiting toxicity revealed in clinical studies was severe nausea and vomiting (regardless of dose or administration route) which were mitigated somewhat by giving the drug in divided doses.[9,11] Nevertheless, the high frequency among patients of nausea and vomiting dimmed the clinical future of this otherwise promising drug.[11] Using a technique of continuous iv infusion of the drug over a 5-day period, however, virtually eliminated the nausea and vomiting toxicity[13,14] while preserving the full therapeutic effect.[13] Although continuous infusion of 1 provides an effective resolution of a toxicity problem, the instability of 1 with respect to hydrolysis adds a complicating factor not important in single dose administration.

An early report from the Czech group[15] described the hydrolysis of 1 in aqueous solutions (see Scheme I) which leads to a guanylurea ribose (3) via an unstable intermediate (2) at a rate which is dependent on pH and temperature.[16] The best clinical formulation of 1 for maximum stability[17] utilizes lactated Ringer's solution for reconstitution of a clinical vial, but even so, 15% of the drug is lost after 4.7 h, when stored at 25 °C, to products of unknown therapeutic value and unknown toxicity.[18] As a consequence, strict control over dosages and drug purity using the continuous infusion technique is difficult to achieve even if the drug is freshly prepared at 4-h intervals as has been recommended.[13]

Scheme I



It was our intent to modify the structure of 1 so as to (i) overcome the water instability problem while (ii) preserving the same antitumor action of the parent. These goals would be ideally achieved using the prodrug concept[19] of drug modification such that the clinical experience gained with 1 would be largely applicable to a solution stable analogue.

We would like to report the synthesis of 5,6-dihydro-5-azacytidine, which is stable for weeks at room tem-

perature in aqueous solution over a broad pH range, and which has substantial activity in mouse leukemia test systems.[20]

Chemistry. The electron density at the 6-carbon atom of the triazine ring system of 1 is significantly lower than in cytosine as shown by quantum chemical calculations.[15] This finding is consistent with the observation that 1 is vulnerable to nucleophilic attack at position 6 which, when the nucleophile is water, results in the fission of the triazine ring with the formation of intermediate 2. A reasoned approach toward making a hydrolytically more stable analogue of 1 would either impede nucleophilic attack on carbon 6 or increase the electron density at that position. For our initial effort we chose the latter alternative through the simple expediency of saturating the 5,6 double bond of 1. While oxidation of the reduced nucleoside to 1 in vivo is a stringent requirement, it could conceivably be accomplished by enzymatic hydroxylation at the C-6 position of the triazine followed by loss of the elements of water to give the aromatic system.

The synthesis of 5,6-dihydro-5-azacytidine hydrochloride (5, Scheme I) was accomplished via a sodium borohydride reduction of 1 in hexamethylphosphoramide (HMPA) solution. The reduction provided an intermediate, boron-containing complex which could be purified by recrystallization from water giving a material that appeared homogeneous by TLC. However, elemental analysis gave variable results but did consistently suggest the presence of one boron atom for every two nucleoside units. By contrast, reduction of 5-azacytosine (11) with borohydride in the same way did *not* result in a boron-containing product but rather gave the reduced base directly. The UV spectrum of the boron complex was similar to that of 5 when measured in two different buffers indicating the lack of involvement of boron with the triazinone chromophore. Structure 4 is proposed for the complex wherein the *cis*-glycol functions of two ribosyl groups participate in a borate anion. Similar borate complexes of ribonucleosides have been used as protecting groups in synthetic schemes.[21]

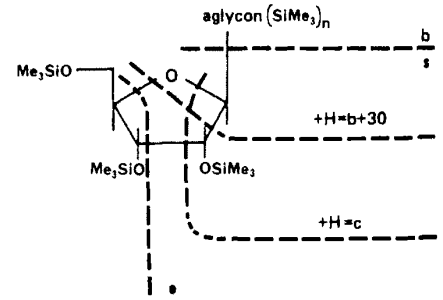
Acid hydrolysis of the complex (4) liberated the reduced nucleoside as the hydrochloride salt (5) which could be converted to the free base (6) either with an anion-exchange column or by treatment with ammonium hydroxide solution. The NMR spectrum of 5 showed the anomeric proton as a doublet at δ 5.54 and a singlet at higher field (δ 4.69) due to the two methylene protons at C-6; the aromatic proton shown by 1 at δ 8.7 was absent. Substitution of sodium borodeuteride for borohydride in the reduction of 1 gave 7 after mild acid hydrolysis. The NMR spectrum of 7 was identical with that of 5 except the singlet due to the C-6 methylene group integrated for only one proton.

Vigorous treatment of 5 with 6 N hydrochloric acid caused cleavage of the glycosidic bond allowing isolation of the aglycon as the hydrochloride salt (9). The salt was converted to the free base (10) using an anion-exchange column. Reduction of 5-azacytosine (11) with sodium borohydride in HMPA solution gave 10 which showed a singlet (δ 4.54) in the NMR spectrum as the only resonance signal due to nonexchangeable protons. The hydrochloride salt (9) was prepared by treatment of 10 with ethanolic hydrogen chloride. Mixture melting points of 9 and 10 with the corresponding reduced triazines obtained from the hydrolysis of the nucleoside (5) gave no depression.

After rendering sufficiently volatile by trimethylsilylation with bis(trimethylsilyl)trifluoroacetamide (BSTFA),[22] the triazine aglycons (10 and 11), the triazine nucleosides (1 and 6), and the deuterated nucleoside (8) were analyzed via gas chromatography–mass spectrometry (GC–MS). 5-Azacytosine (11) as the bis(trimethylsilyl) derivative gave an isothermal retention index[23] (IRI) of 1480 in the GC (3% SE-30 liquid phase) and the mass spectrum showed an intense parent ion at m/e 256. In the case of dihydro-5-azacytosine (10) the molecular ion (m/e 330) is quite diagnostic since reduction of the 5,6 double bond of 11 produces an additional site for trimethylsilylation. Loss of hydrogen from the molecular ion is also much enhanced since it results in formation of an aromatic immonium ion. The IRI of 10 as the tris(trimethylsilyl) derivative was 1670.

Silylation of 5-azacytidine (1) with BSTFA smoothly gave a tetrakis(trimethylsilyl) derivative that gave a single peak in the GC (IRI 2620) and gave a peak for the molecular ion at m/e 532. The reduced nucleoside (6) has an additional site subject to trimethylsilylation which leads to a pentakis(trimethylsilyl) derivative (IRI 2465) exhibiting a peak at m/e 606 due to the molecular ion. Selected ions from the mass spectra of the derivatized nucleosides, which are compared in Table I, possess several sets of diagnostic ions:[24,25] (i) ions resulting from loss of neutral species from the intact molecular ion such as the M – CH_3 ion, (ii) ions resulting from the derivatized base (b) and its rearrangements, and (iii) ions resulting from the trimethylsilylated ribosyl moiety(s). All the ions produced by derivatized 6 which are associated with the triazine nucleus attest to its reduced nature. The deuterated nucleoside (8) from $NaBD_4$ reduction when trimethylsilylated with BSTEA gives a molecular ion one atomic mass unit higher (m/e 607) than the corresponding nondeuterated material, and the shift of all base series ions (b, b + H, b + 2H, b + 30, c, e) one unit higher indicates incorporation has occurred in the triazine moiety of the nucleoside.

Table I. Mass Spectrometry Fragmentation Patterns for Silylated 5-Azacytidine (1) and Dihydro-5-azacytidine (6)



Ion	m/e (rel intensity) 5-Azacytidine·4Me_3Si	Dihydro-5-azacytidine·5Me_3Si
M^+	532 (0.14)	606 (1.2)
M – H		605 (1.6)
M – 15	517 (0.77)	591 (1.6)
e	314 (0.82)	388 (2.4)
c	299 (0.38)	373 (1.7)
s – H	348 (3.4)	348 (3.0)
b + 30		287 (2.3)
b + 2H	185 (3.1)	259 (8.8)[a]
b		257 (9.7)
Base peak	73 (100)	73 (100)

[a] This mass also represents the loss of Me_3SiOH from the intact sugar fragment as well as the +1 isotope peak for the isobaric b + H and s – H – Me_3SiOH ions. Analysis of the mass spectrum of trimethylsilylated 8 where the b + 2H peak occurs at m/e 260 indicates that the b + 2H ion is only responsible for a minor fraction of this peak.

Table II. Comparative Survival Times of Mice Treated with 5-Azacytidine (1) or Dihydro-5-azacytidine (5) in Antitumor Test Systems[a]

System no.	Tumor	Schedule[b]	Compd	Expt no.[c]	Dose[d] range	% ILS at optimal dose	Min active dose	Optimal active dose	Max active dose	T − C[e]
1	L1210	QD1	5	8891	300-1600	34	300	600	1600	−3.1
				8892	300-1600	39	300	1600	1600	−1.5
			1	8891	12.5-100	73	12.5	50	100	−3.5
				8892	12.5-100	75	12.5	50	100	−3.8
2	L1210	Q4D	5	8887	150-600	104	150	600	600	−4.3
				8895	12.5-800	96	12.5	800	800	−1.5
			1	8887	5-40	138	5	20	40	−1.2
				8895	5-40	94	5	10	20	−1.5
3	L1210	Q4D(Q6H)	5	8891	9.4-150	68	9.4	150	150	−1.5
				8892	9.4-150	72	18.7	125	150	−2.5
			1	8891	1.25-10	126	1.25	5	5	−0.5
				8892	1.25-10	135	1.25	5	5	−1.5
4	L1210	QD1-9	5	8892	6.25-400	116	6.25	100	300	−3.5
				R002	0.78-600	128	3.13	100	200	−3.9
			1	8892	0.75-6	128	0.75	3	6	0.9
				R002	0.78-12.5	172	0.78	3.13	3.13	−3.0
5	L1210/AZA	QD1-9	5	01	0.78-600	9		25		0.1
				02	0.39-200	0				
			1	01	0.78-12.5	0				
				02	0.78-12.5	0				
6	PO-L1210	QD1-9	5	8903	12.5-1000	50	200	400	400	−4.4
				8904	12.5-1000	82	400	1000	1000	−2.2
			1	8903	1.56-25	24		25		−4.1
				8904	1.56-25	125	1.56	12.5	25	−3.1
7	IC-L1210	QD1-9	5	47	6.25-400	46	25	100	100	−1.9
				48	6.25-400	44	25	100	200	−0.8
			1	47	0.75-6	27	0.75	0.75	0.75	0.7
				48	0.75-6	28	0.75	0.75	0.75	−0.5
8	P388	QD1-9	5	5768	1.56-400	85	12.5	100	200	−2.8
				3968	0.75-600	100	12.5	200	200	−4.3
			1	5768	0.75-6	127	0.75	3	3	−0.4
				3968	0.75-12.5	162	0.75	3.13	3.13	−4.3
9	B16	QD1-9	5	326	1.56-400	19		200		−3.3
				327	1.56-400	25		100		−2.0
			1	326	0.75-6	20		3		−1.5
				327	0.75-6	42	1.5	3		−0.5

[a] Test compounds in 0.9% saline solution were administered intraperitoneally (ip) according to the specified schedule except in the PO-L1210 system (system 6) where the compounds were administered orally to nonfasting animals. The mice were tumored ip except in the IC-L1210 system (system 7) where tumor cells were implanted intracerebrally (ic). [b] In the QD1 schedule drug was administered only on the first day after tumor implantation (one injection). Drug was given on days 1, 5, and 9 with the Q4D schedule (three injections) and on days 1-9 with the QD1-9 schedule (nine injections). The Q4D(Q6H) schedule required drug administration every 6 h on days 1, 5, and 9 (12 injections). [c] Where more than two experiments were carried out for a compound in a specific system, the two experiments which produced the highest ILS values were selected for tabulation. [d] All doses are expressed in milligrams per kilogram of body weight per injection. [e] The difference of the average body weight change in grams of the optimal dose level test group (T) and the control group (C) measured on day 5.

Antitumor Evaluations. Dihydro-5-azacytidine hydrochloride (**5**) was evaluated for antitumor activity in comparative studies (Table II) with 5-azacytidine (1) according to protocols[26] devised by the Division of Cancer Treatment, National Cancer Institute, using the following mouse tumor test systems: lymphoid leukemia L1210, lymphocytic leukemia P388, and melanotic melanoma B16. In each system dose–response assays were conducted where each successive dose was half of the preceding higher dose. The increase in life-span of the test animals beyond the survival time of the untreated control animals expressed as a percentage (% ILS) was used in all the test systems to evaluate antitumor activity. Activity is defined here as a % ILS value $\geq$25% for L1210 and P388 and $\geq$40% for B16.

The L1210 assay, which was most sensitive to **5**, was used to elucidate several questions. Systems 1–4 (Table II) sought to reveal a possible schedule dependency on the activity of **5** when administered intraperitoneally (ip). The effectiveness of oral administration of **5** was explored in system 6 (PO-L1210). To probe the ability of **5** to cross the blood–brain barrier, system 7 (IC-L1210) was employed wherein tumor cells were implanted in the cranial cavity and drug was administered ip. An L1210 subline resistant to 5-azacytidine (L1210/AZA) was used to test (system 5) a possible cross resistance of the dihydro analogue with the parent.

The P388 tumor was used to assess the activity of **5** against a second leukemia model and the B16 test system was utilized to uncover any potential activity of **5** in a solid tumor model (systems 8 and 9, respectively).

Results and Discussion

Of the two objectives set forth for this study (vide supra), the first has been clearly met. We have reported[20] that buffered solutions (pH 2 and 6) of dihydro-5-azacytidine (**5**) showed no decomposition at 25 °C over a 3-week observation period as determined by a quantitative NMR assay. Moreover, **5** can be isolated unchanged (as the free base) after storing for 7 days at room temperature in 1 N ammonium hydroxide solution. Thus, the reduced nucleoside (**5**) is completely stable in aqueous solutions allowing, if necessary, administration by the continuous infusion technique without concern for drug hydrolysis in

Table III. L1210 Leukemia Antitumor Evaluation of Incidental Compounds[a]

Compd	Schedule	Dose range, mg/kg	% ILS[b] (dose)	T − C[c] (dose)
3	Q4D	5-365	0 (240)	−2.2 (240)
4	Q4D	25-400	80 (400)	−1.5 (400)
6	QD1-9	1.56-400	86 (50)	−3.2 (400)
9	QD4	6.25-400	0 (400)	1.0 (400)

[a] Test compounds in 0.9% saline solution were administered intraperitoneally (ip) according to the specified schedule following ip tumor implantation. [b] The tabulated dose (mg/kg) was the highest nontoxic dose given producing the indicated % ILS in a dose-response assay or the dose producing the maximum % ILS in the case of active compounds. [c] The difference of the average body weight change in grams of the test group (T) and the control group (C) measured on day 5 at the highest nontoxic dose tested.

aqueous formulation. By comparison, the parent drug (1) has a half-life of 48 h at pH 7 (25 °C).[18] Moreover, it is completely decomposed in a few hours in 1 N ammonium hydroxide solution giving mainly a guanylurea ribose (3), which is considerably less inhibitory to the growth of *Escherichia coli* than 1,[15] and as the picrate salt, has no in vivo antitumor activity against L1210 leukemia (Table III).

Several salient points of interest can be derived from systems 1–4 of the comparative L1210 testing outlined in Table II. First, although both drugs exhibit good activity, the efficacy of **5**, in the main, is somewhat less than that of 1. Second, the dose level of **5** necessary to produce maximum activity is about 35 times the optimum dose of 1. This is most clearly in evidence when the results of the QD1–9 schedule (system 4) are considered. The apparent potency differential could also be explained by a 1–3% contamination of the reduced nucleoside (**5**) by the parent drug (1) which served as a starting material. However, using a high-pressure liquid chromatography analytical method the concentration of 1 (if present at all) in samples of **5** was shown to be considerably less than 0.05%[20] and would not contribute to the observed antitumor response of **5**. When **5** was stored overnight in 1 N ammonium hydroxide solution at room temperature the free base (**6**) thus obtained exhibited antitumor activity (Table III) within the anticipated % ILS range. Since 1 is readily decomposed by ammonium hydroxide, one could assume its selective destruction if it were present in **5** so treated. Therefore, it can be concluded that there is no detectable **1** in the reduced nucleoside, and **5** is solely responsible for the antitumor activity observed. Third, the activity of both **1** and **5** is not very sensitive to changes in administration schedules. This is a somewhat surprising observation since it has been reported[27,28] that 1 appears to be cell cycle specific to cultured L1210 cells. Initial testing results led us to believe **5** was rapidly excreted due to its good water solubility (40 mg/mL, pH 6) so that if the drug were administered every 6 h on days 1, 5, and 9, an improvement in % ILS might be realized. In addition, if there were some cell cycle specificity, the Q4D(Q6H) schedule might better be able to exploit it than the Q4D regimen. On the contrary, the more intensive schedule (system 3) appeared to be less effective.

The data from the two L1210 QD1–9 experiments (system 4) presented in abbreviated form in Table II were used along with data generated from three additional L1210 QD1–9 experiments determined during the course of this study to develop dose–response relationships for **1** and **5** in order to calculate their respective therapeutic indices (TI). Using the method of Skipper and Schmidt[29] (TI = optimal dose for maximum % ILS/lowest dose giving 40% ILS) one obtains TI = 10 for **5** (100 mg/kg ÷ 10 mg/kg) and TI = 7.5 for 1 (3 mg/kg ÷ 0.4 mg/kg). Alternatively, the method of Goldin[30] (TI = highest dose giving 40% ILS/lowest dose giving 40% ILS) yields TI = 40 for **5** (400 mg/kg ÷ 10 mg/kg) and TI = 14 for 1 (5.7 mg/kg ÷ 0.4 mg/kg). Therefore, it appears that **5** has a more favorable therapeutic index than 1 as found by two methods of calculation.

The remaining portion of Table II gives the results of some special studies done with the L1210 model (systems 5–7) as well as the testing results of **5** using two other tumor models (systems 8 and 9).

In an attempt to approach the question of a possible prodrug nature of **5**, a subline of L1210 leukemia which is resistant to 1 was used to establish a possible cross resistance between 1 and **5** (system 5, L1210/AZA). In comparative experiments the L1210/AZA tumor was completely resistant to both **1** and **5**, a result which is consistent with **5** being a prodrug of 1, although the results could also be explained in terms of similar mechanisms of drug action.

Unlike 1, the dihydro analogue (**5**) is stable to hydrolysis at pH 2 and would be capable of withstanding acidic stomach conditions making oral administration a possible route. Therefore, **5** was tested in L1210 where drug was given orally to the test animals (system 6, PO-L1210). Reproducible activity was observed which suggests the feasibility of this administration route in clinical usage, perhaps as part of remission maintenance therapy. Although 1 was found to be marginally active in this system, its known instability in the low pH[15,16] range would probably preclude any serious consideration for clinical use using the oral administration route.

Implantation of L1210 cells intracerebrally (system 7, IC-L1210) provided a good model to determine the ability of **5** to cross the blood–brain barrier. In this test **5** was clearly superior to 1 in terms of the % ILS parameter. 1 is known to penetrate only slightly into the central nervous system (CNS)[31] and therefore is unable to reach the tumor cells in effective concentration. The reproducible activity of **5** in the brain tumor model might serve as an indicator of a special advantage it might have relative to 1 in the clinical treatment of leukemia. Infiltration of the CNS by leukemic cells occurs in a high percentage of leukemic children, and incidence increases with increasing survival time of the child. Accordingly, intrathecal administration of cytostatic agents and/or radiation usually constitutes part of treatment whether or not CNS involvement is diagnosed.[32] It is possible **5** could find a place in the treatment of leukemia where it not only treats the primary disease site but also provides prophylaxis for the CNS.

Antitumor testing of **5** was extended to P388 leukemia (system 8) and the solid tumor model, B16 melanoma (system 9). As was anticipated, **5** had good activity against the P388 system. On the other hand, both **1** and **5** were essentially inactive against the murine solid tumor which is commensurate with the clinical trials[11,12] of 1 in treatment of solid tumors.

In addition to the in vivo comparative bioassays of 1 and **5** described above, comparative cytotoxicity studies on cultured L1210 cells have also been conducted.[33] Solutions of **5** were found to be cytotoxic but required a tenfold higher concentration than that found necessary for 1 to cause cessation of growth. Protection against the effects of 1 or **5** is afforded the L1210 cells by either cytidine or

uridine added to the culture medium at the same time as the drug. While the in vitro cytotoxicity of **5** is difficult to rationalize in terms of a prodrug role for **5** where metabolic activation is required, **5** may be intrinsically cytotoxic. On the other hand, the following subjective evidence indicates that the dihydro analogue may be a prodrug form of 5-azacytidine: (a) both are active in the same murine leukemia test systems but neither is active against the B16 solid tumor; (b) neither has any apparent schedule dependency; and (c) in experiments with the L1210/5-azacytidine resistant tumor, a cross resistance was observed. Definitive information to resolve the prodrug hypothesis will best come from in vitro and in vivo metabolite studies.

Table III gives the antitumor data for some synthetic intermediates and other compounds peripheral to this study. There was occasion to discuss compounds **3** and **6** above. Compound **4**, which is the boron-containing intermediate from the borohydride reduction of **1**, has activity in L1210 leukemia which approximates the level of activity shown by **5** in the same system (Table II, system 2). The most probable explanation for this result involves in vivo hydrolysis of the borate ester to give **5** which acts by itself or through a metabolic product to exert the observed antileukemic effect. In view of the potential use of boron-containing compounds in cancer therapy of the CNS,[34] **4** was tested in the murine ependymoblastoma brain tumor model.[35,36] The results, however, were negative perhaps due to the inability of a charged molecule to penetrate the blood–brain barrier in sufficient quantity.

Conclusion

It is felt that dihydro-5-azacytidine (**5**) has sufficient merit as a potential antitumor drug to warrant further preclinical study. Formulation should cause a minimum of difficulty due to the good water solubility of **5** and its stability over a broad pH range. Although the dose levels necessary for optimal effects are high relative to **1**, and might prove to be disadvantageous, the antitumor activity of **5** is, nevertheless, quite substantial in the L1210 model which has been used as an indicator system for clinical effectiveness.[37] The stability of the reduced nucleoside to acid in addition to its demonstrated activity in L1210 leukemia via oral administration might constitute an advantage in clinical usage over the parent drug. Similarly, the activity of **5** in the IC-L1210 model perhaps signals a potential CNS prophylaxis role in the treatment of acute leukemia. Finally, the more favorable therapeutic index of **5** suggests it might be used with a greater margin of safety than **1**.

Experimental Section

The GC–MS system consisted of a Varian Aerograph 2740 gas chromatograph coupled to a Du Pont 21-492 mass spectrometer via a glass transfer line and jet separator maintained at 255 °C. The mass spectrometer was operated with the ion source at 240 °C, a 300-μA ionizing current, and a 75-eV ionizing voltage. The injector port of the chromatograph was equilibrated at 250 °C and the flame ionization detector (FID) was maintained at 275 °C. A 5 ft × $^1/_8$ in. stainless steel column packed with 3% SE-30 on 120 mesh Variport Q was operated isothermally at 220 °C. The GC column effluent was directed to both the mass spectrometer ion source and FID through a fixed-ratio split so that a gas chromatogram, a total-ion current chromatogram, and a mass spectrum could be recorded simultaneously. Proton NMR spectra were recorded with a Varian T-60 or a Varian HA-100D spectrometer in Me_2SO-d_6 or D_2O solution using Me_4Si or TSP, respectively, as internal standards. When required, labile protons were completely exchanged by dissolving the samples in D_2O and lyophilizing the solution. Repeating the D_2O dissolution and lyophilization a total of three times gave the NMR sample. UV spectra ($\epsilon \times 10^{-3}$) were recorded on a Cary Model 15 spectrometer using pH 2 (potassium chloride–hydrochloric acid) or pH 8 (monobasic potassium phosphate–sodium hydroxide) buffers. Specific rotations were measured in a 1-dm cell with a Perkin-Elmer Model 141 polarimeter. Elemental analyses were carried out by the Section on Microanalytical Services and Instrumentation, NIAMDD, NIH, and by Galbraith Laboratories, Inc., Knoxville, Tenn. Where analyses are reported only by the symbols of the elements, results were within ±0.4% of the theoretical values. Compound purity was routinely checked by TLC using 5 × 20 cm plates coated with Baker 1B2-F silica gel. Four solvent systems were employed: butanol–ethanol–water (40:11:19), butanol–acetic acid–water (5:2:3), 2-propanol–ammonia–water (7:1:2), isobutyric acid–ammonia–water (66:33:1.5). Spot visualization was achieved with UV light or by charring after spraying the plate with concentrated sulfuric acid. Melting points were determined with a Thomas-Hoover capillary apparatus and are uncorrected.

1-β-D-Ribofuranosyl-3-guanylurea (3). The procedure of Pithova et al.[15] was followed for the hydrolysis of **1** with 1 N aqueous ammonia solution to give **3** as a syrup. When a water solution of the syrup was lyophilized a fluffy white material was obtained which was very hygroscopic: mp 103–105 °C dec (sinters at 82–86 °C).

A methanol solution of the syrup gave the picrate in 70% yield when treated with ethanolic picric acid: mp 172–174 °C (lit.[15] 172–174 °C). The melting point was raised to 177–178 °C by recrystallization from methanol. Anal. ($C_7H_{14}N_4O_5 \cdot C_6H_3N_3O_7$, 463.3) C, H, N.

4-Amino-5,6-dihydro-1-β-D-ribofuranosyl-1,3,5-triazin-2(1*H*)-one Hydrochloride (5). A solution of 4.88 g (0.02 mol) of **1** in 20 mL of HMPA was treated with 1.52 g (0.04 mol) of sodium borohydride and stirred at 50 °C for 1 h and then at room temperature for 3 h. The reaction was hydrolyzed with 50 mL each of methanol and water and allowed to stand at 0 °C overnight. Concentration of the solution under vacuum (bath 30 °C) gave a syrup which was washed with ether and the residue taken up in 70 mL of methanol. The boron complex (**4**) as a white powder (3.81 g) was precipitated by careful addition of ether (70 mL). Evaporation of the supernatant and subjecting the residue to another ether–methanol precipitation provided an additional 0.33 g of **4**: total yield 79%; mp >330 °C (darkens at 260 °C). Recrystallization could be effected from concentrated water solutions. In large-scale preparations the combined crops from ether–methanol precipitation are best recrystallized from water after adjusting the pH to 7 with a few drops of dilute hydrochloric acid: UV λ_{max} (pH 2) end absorption; UV λ_{max} (pH 8) 233 nm (7.37).

A solution of **4** (4.60 g, 0.017 mol) in 40 mL of 6 N hydrochloric acid was stirred at 21 °C for 4 h. Ethanol (70 mL) was added and stirring continued at −10 °C for 1 h to give a white precipitate which was removed by filtration and washed successively with ethanol and ether. On standing the reaction solution gave a second crop bringing the total yield of **5** to 4.05 g (85%): mp 180–181 °C dec. Recrystallization from methanol–ethanol gave colorless needles: mp 180–181 °C dec; $[\alpha]^{26}_D$ −29° (*c* 1.0, H_2O); UV λ_{max} (pH 2) end absorption; UV λ_{max} (pH 8) 233 nm (4.98); NMR (Me_2SO-d_6) δ 5.54 (d, J = 6 Hz, 1, C_1·H), 4.69 (s, 2, C_6H). Anal. ($C_8H_{14}N_4O_5$·HCl, 282.7) C, H, N, Cl.

4-Amino-5,6-dihydro-1-β-D-ribofuranosyl-1,3,5-triazin-2(1*H*)-one (6). A solution of 1.50 g (5.3 mmol) of the hydrochloride **5** in 150 mL of 1 N ammonium hydroxide was stirred at room temperature for 5 h, stored overnight at 25 °C, and then concentrated under vacuum (25 °C bath) to ca. 20 mL. Addition of 100 mL of absolute ethanol to the concentrate gave 1.15 g (89%) of crystals which when recrystallized from absolute ethanol provided 0.82 g of **6**, mp 216–218 °C dec. The analytical sample (methanol) melted at 218–220 °C dec: $[\alpha]^{29}_D$ −23° (*c* 1.0, H_2O); UV λ_{max} (pH 2) end absorption; UV λ_{max} (pH 8) 233 nm (6.69); NMR (Me_2SO-d_6) δ 5.59 (d, J = 6 Hz, C_1·H), 4.57 (s, 2, C_6H); NMR (D_2O) δ 5.75 (d, J = 6 Hz, 1, C_1·H), 4.64 (s, 2, C_6H). Anal. ($C_8H_{14}N_4O_5$, 246.2) C, H, N.

The free base (**6**) could also be formed by passing an aqueous solution of **5** slowly through a Dowex 1-X2 (OH^- form) column. Lyophilization of the aqueous eluates and recrystallization from methanol gave **6** in good yield: mp 218–219 °C.

Reduction of 1 with Sodium Borodeuteride. 4-Amino-5,6-dihydro-1-β-D-ribofuranosyl-1,3,5-triazin-2(1*H*)-one-6-*d* Hydrochloride (7). A solution of **1** (976 mg, 4.0 mmol) in 5 mL of HMPA was reduced with 168 mg (4.0 mmol) of sodium borodeuteride using the procedure described above for the preparation of **5**. The boron complex, thus obtained, was recrystallized from water to give 887 mg of white crystals, mp >300 °C. The complex was hydrolyzed with 6 N hydrochloric acid (6 mL) for 4 h at room temperature as before affording 570 mg of **7** after recrystallization from ethanol: mp 180–181 °C dec.

The free base (**8**) was liberated from an aqueous solution of **7** (220 mg) run through a Dowex 1-X2 (OH^- form) column. After lyophilization of the eluates and recrystallization from ethanol, 145 mg of **8** was obtained: mp 218–220 °C. The integrated NMR spectrum was best observed free from the interference of the signal due to traces of water. To that end 25 mg was silylated in an NMR tube with 0.6 mL of a BSTFA–acetonitrile-d_3 solution (1:2) by sonication at room temperature until solution was complete. The observed integrated areas were 1:1:5 for the anomeric proton, the C-6 methylene proton, and the ribosyl protons, respectively.

Hydrolysis of 5. 5,6-Dihydro-5-azacytosine Hydrochloride (9). A solution of **5** (141 mg, 0.5 mmol) in 5 mL of 6 N hydrochloric acid was heated on a steam bath for 30 min. The darkened reaction solution was treated with charcoal and filtered. Concentration of the filtrate under reduced pressure to approximately 1 mL caused crystals (50 mg, 66%) to separate which were washed with absolute ethanol, mp 255–260 °C dec. Recrystallization from ethanol (10 mL) raised the melting point to 259–261 °C dec. Mixture melting point with **9** prepared by reduction of **11** (see below) gave no depression and spectral properties were identical.

A methanol solution of **9** from the nucleoside hydrolysis when passed through a Dowex 1-X2 column (OH^- form) gave the free base (**10**): mp 186–188 °C dec. Mixture melting point with **10** prepared from **11** was 188–189 °C dec.

5,6-Dihydro-5-azacytosine (10) from the Reduction of 11. To a solution of 5-azacytosine (**11**, 1.12 g, 10 mmol) in 7 mL of HMPA was added, with stirring at room temperature, 1.0 g (26 mmol) of sodium borohydride in small portions over 15 min. The reaction mixture was stirred at 21 °C for 1 h and then heated at 50 °C for 2 h. After storing the solution overnight at room temperature, 10 mL of methanol was added (stirred 2 h), followed by 10 mL of water. The following day solvents were removed in vacuo (30 °C bath) to afford a syrup which was triturated successively with portions of ether and portions of absolute ethanol. A solid separated (0.9 g) which was recrystallized three times from absolute ethanol giving 0.4 g (35%) of **10** as white crystals: mp 189–190 °C dec; UV λ_{max} (pH 2) end absorption; UV λ_{max} (pH 8) 233 nm (4.14); NMR (D_2O) δ 4.54 (s, C_6H); GC–MS (trimethylsilyl derivative) *m/e* (rel intensity) 330 (12), 329 (25), 257 (28), 256 (13), 241 (12), 171 (100), 142 (9), 100 (57), 99 (28). Anal. ($C_3H_6N_4O$, 114.1) C, H, N.

The starting material (**11**) gave UV λ_{max} (pH 2) 246 nm (5.13); UV λ_{max} (pH 8) 245 nm (3.54, shoulder); GC–MS (trimethylsilyl derivative) *m/e* (rel intensity) 256 (98), 255 (2), 214 (5), 198 (3), 171 (100), 142 (34), 100 (33), 99 (50).

The hydrochloride salt of **10** was prepared by treating a solution of **10** (30 mg, 0.26 mmol) in absolute ethanol (5 mL) with 2 mL of absolute ethanol saturated with gaseous hydrogen chloride. The reaction solution was warmed at 50 °C for 10 min and then allowed to cool to room temperature causing the salt (**9**) to crystallize from solution (30 mg, 77%): mp 259–261 °C dec. Recrystallization from absolute ethanol gave the analytical sample: mp 260–261 °C dec; NMR (D_2O) δ 4.65 (s, C_6H). Anal. ($C_3H_6N_4O$·HCl, 150.6) C, H, N, Cl.

Silylation Procedure. Nucleosides and their aglycons (**1**, **6**, **8**, **10**, and **11**) were derivatized for gas chromatography (GC) and combined gas chromatography–mass spectrometry (GC–MS) by trimethylsilylation with bis(trimethylsilyl)trifluoroacetamide (BSTFA). A 1:2 solution of BSTFA–acetonitrile was allowed to react with 1–3 mg of the appropriate free base in a 14 × 48 mm screw cap vial with a Teflon-lined rubber septum by sonication at room temperature until solution occurred (usually 10–15 min). Aliquots of these solutions were used directly for analysis. In the case of **6**, **8**, and **10**, heating the silylation mixture initiated an oxidative decomposition of the trimethylsilylated derivative to an extent which was proportional to temperature and duration of heating.

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Synthesis and Antileukemic Activity of 5-Substituted 2,3-Dihydro-6,7-bis(hydroxymethyl)-1*H*-pyrrolizine Diesters

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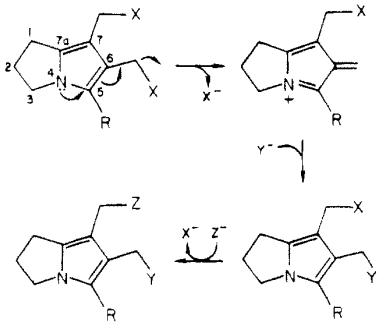
Treatment of *N*-acylproline derivatives, **2**, with acetic anhydride–dimethyl acetylenedicarboxylate (DMAD) gave 5-substituted derivatives of dimethyl 2,3-dihydro-1*H*-pyrrolizine-6,7-dicarboxylate (**5**). The reaction proceeds via a 1,3-dipolar addition of DMAD with the mesoionic oxazalone intermediate **3**, generated in situ, with concomitant elimination of carbon dioxide. Reduction of **5** gave the diols **6** which upon subsequent acylation gave **1**. The bis(*N*-methylcarbamate) **1d** and the diacetate **1i** show a modest level of in vivo antileukemic activity in the L1210 assay. A majority of the diesters, **1**, showed significant antileukemic activity in the in vivo P-388 assay. The bis(carbamate) **1d** afforded "cures" at dose levels as low as 12.5 mg/kg; **1q** showed potent activity at doses as low as 0.78 mg/kg. Several other compounds showed potent activity against P-388 over a greater than fourfold dose range with no acute toxicity. Half-lives for several diacetate derivatives of **1** were determined for aqueous Me_2SO solutions. The preparation of **7** and **8** shows that **1** may react by *O*-alkyl ester cleavage.

A large number of structurally diverse naturally occurring tumor inhibitory compounds have been isolated and identified over the past several years and a major proportion of these compounds contains at least one, often two or three, reactive electrophilic centers in the molecule in addition to several nonelectrophilic moieties.[1] The polyfunctionality is significant because it is the complex interrelationship of these functional groups that contributes to the antitumor activity, cell specificity, and toxicity which these compounds exhibit overall.[2]

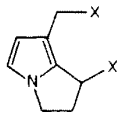
Structure–activity relationship studies with many of these natural products are often limited by the small quantities of material available and by the relatively limited number of modifications which actually can be performed on these complex molecules. Insight into the relationships between structure and activity can, in many instances, only be gained through studies with simpler molecules. The design of these simpler molecules uses the natural product as the base template.[3-5]

During the course of our continuing effort to prepare simple polyfunctional compounds for antitumor evaluation, we synthesized a series of substituted 2,3-dihydro-6,7-bis(hydroxymethyl)-1*H*-pyrrolizine diesters (**1**). Compounds of this type were chosen for study on the basis of certain similarities with the tumor inhibitory mitomycins[6] and pyrrolizidine alkaloids.[7] The pyrrole metabolites of various pyrrolizidine alkaloids can act as alkylating agents but are too reactive and too toxic for drug use. Mitomycin, on the other hand, possesses similar reactive electrophilic centers but is sufficiently stable to reach the cell nucleus where it can react with DNA. Both the mitomycins and

Scheme I[12]



the pyrrolizidine alkaloid pyrrole metabolites possess the general partial structure



Since the pyrrole metabolites of the pyrrolizidine alkaloids appear to be too reactive to give useful cancer chemotherapeutic activity, it should be possible to modulate this reactivity downward toward that of the mitomycins. The potential electrophilic reactivity of the allylic esters in **1** (via *O*-alkyl cleavage[8]) will be enhanced by participation of the ring nitrogen (Scheme I) similar to the mitomycins and pyrrolizidine alkaloid pyrrole metabolites. Fur-

Clinical Trial With Subcutaneously Administered 5-Azacytidine (NSC-102816) [1,2,3]

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SUMMARY

5-Azacytidine was administered subcutaneously (sc) to 22 patients with a variety of advanced solid malignant tumors for 10 successive days to a total dose of 275–850 mg/m². Objective tumor regressions were seen in two patients: one with adenocarcinoma of the breast and one with adenocarcinoma of the ovary. Mild gastrointestinal toxicity was noted at doses producing significant bone marrow depression. Of particular interest was the observation of hepatic toxicity in five patients, three of whom went into hepatic coma. Sc administration of 5-azacytidine is a useful alternative to the standard intravenous route of administration, especially in patients with inadequate sites for venipuncture.

[Cancer Chemother Rep 58:217–222, 1974]

5-Azacytidine is the pyrimidine analog of cytidine with a nitrogen atom substituted for carbon in the 5-position of the ring (fig 1). Píškala and Šorm (1) synthesized this nucleoside in 1964; 2 years later, Hanka et al (2) isolated it from a culture of *Streptoverticillium ladakanus*. 5-Azacytidine has been studied in many in vitro systems and potent antibacterial (3–5), mutagenic (6, 7), and cytostatic (8) activity has been demonstrated. Mechanisms of action of 5-azacytidine include competition for uridine kinase (9), inhibition of orotidylic acid decarboxylase (10), incorporation into RNA (11,12), and incorporation into DNA (12,13). This antimetabolite has shown significant antitumor activity against murine L1210 leukemia, Walker 256 carcinosarcoma,[10] and acute lymphoblastic leukemia in AK mice (14). On the basis of these preclinical studies, 5-azacytidine was chosen for trials in man.

A phase I study of intravenously (iv) administered 5-azacytidine using a total dose of

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[2] Supported by Public Health Service (PHS) grants CA-12291, CA-06071, CA-06551, and CA-05255 (National Cancer Institute [NCI]) and RR-05539 (Division of Research Resources), National Institutes of Health (NIH), Department of Health, Education, and Welfare (DHEW); by contract NIH-70-2136 from the Division of Cancer Treatment (DCT), NCI, NIH, PHS, DHEW; and by an appropriation from the Commonwealth of Pennsylvania.

[3] 5-Azacytidine was supplied by Cancer Therapy Evaluation, DCT, NCI. Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are listed in the List of Compounds at the end of the text.

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[9] We wish to thank Dr. Richard Bornstein for entering patients into this clinical trial.

[10] Broder LE, and Carter SK. Clinical brochure: 5-azacytidine (NSC-102816), Oct 1970, 25 pp. Prepared by the NCI, Bethesda, Md.

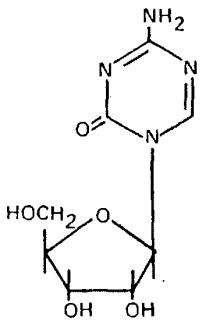

FIGURE 1.—Structure of 5-azacytidine.

300–700 mg/m² revealed that the drug has gastrointestinal and hematologic toxic effects (15). The severe incapacitating emesis resulting from the iv administration of 5-azacytidine and the lack of local tissue reaction upon extravasation provided the impetus for this investigation of an alternate route of administration. Furthermore, in a comparison of iv and subcutaneous (sc) administration of 5-azacytidine (16), plasma levels were not significantly different 1 hour after injection and preferential concentration in tumor tissue was demonstrated regardless of the route of administration.

MATERIALS AND METHODS

5-Azacytidine was administered to 22 patients with a variety of advanced malignant tumors. Histologic evidence of malignancy and a measurable lesion were prerequisites for entry into this study. Patients who were expected to live less than 3 months and patients who had had previous total pelvic irradiation, prior adrenalectomy and/or hypophysectomy, or irradiation or chemotherapy within 30 days of the start of the study were excluded from the study. Also excluded were patients who had a hemoglobin value less than 9 g/100 ml, a white blood cell count (wbc) less than 4000 cells/mm³, a platelet count less than 100,000 platelets/mm³, a blood urea nitrogen (BUN) value greater than 25 mg/100 ml, a bilirubin value greater than 1.5 mg/100 ml, or a serum glutamic oxaloacetic transaminase (SGOT) value greater than 120 International Units (IU) (normal value, 5–40 IU).

Eight men and 14 women were included in this study. The mean age of the men was 63 years (range, 49–77 years) and the mean age of the women was 58 years (range, 33–74 years) (table 1). Patients were treated with 5-azacytidine at a dose of 275–850 mg/m² given sc for 10 successive days as initial therapy. Patients who responded to initial therapy received maintenance treatment with 5-azacytidine at a dose of 35–90 mg/m²/week.

Palpable lesions were measured weekly. Pulmonary parenchymal masses were evaluated with weekly chest X-ray films and hepatic lesions were evaluated with biweekly liver scans.

Hemoglobin determinations, wbc counts, and platelet counts were performed on Days 1, 5, and 10 of therapy and thereafter at weekly intervals. BUN, bilirubin, and SGOT values were determined weekly.

5-Azacytidine was supplied in sterile vials containing 50 mg of white lyophilized powder. The contents of each vial were reconstituted with 3 ml of sterile water immediately prior to injection.

RESULTS

Toxicity

Four patients died of progressive disease prior to the completion of the 6-week followup period and are excluded from the toxicity evaluation. The toxicity experienced by the 18 evaluable patients is summarized in table 2. In six patients there were no toxic effects; in 12 patients definite single or multiple toxic effects occurred. One patient had severe stomatitis. No dermatitis, alopecia, or renal impairment was noted.

Twelve patients experienced anorexia during the 10-day loading regimen; eight of these patients had nausea, which was generally well tolerated. Only four patients experienced drug-related vomiting. Vomiting began 2–3 hours after injection, was controlled by moderate doses of antiemetics, and cleared in 3–4 hours. Tolerance to nausea and vomiting developed in all patients as additional doses of 5-azacytidine were administered so that by Day 10 of the loading regimen no vomiting was noted. Three patients had drug-related episodes of diarrhea which were self-limited and mild.

Deterioration in liver function was seen in

TABLE 1.—Summary of data for evaluable patients treated with 5-azacytidine

Patient No.	Age,sex	Tumor	Prior therapy*	Total dose of 5-azacytidine	
				Mg/m²	Mg
1	71,F	Ovary	S, cyclophosphamide, adriamycin	275	400
2	62,F	Ovary	S	345	500
3	61,F	Ovary	S, R, cyclophosphamide	370	500
4	64,F	Breast	S, R, cyclophosphamide	385	500
5	63,F	Tonsil	S, R	417	500
6	56,M	Lung	S, cyclophosphamide, methotrexate	333	525
7	74,F	Breast	S, R	375	600
8	49,M	Hepatoma	S, 5-FU	441	750
9	51,F	Ovary	S	500	750
10	67,M	Colon	S, R, 5-FU	513	1000
11	58,M	Hypernephroma	S, hydroxyurea, adriamycin	587	1000
12	63,M	Melanoma	S, NSC-45388	588	1000
13	67,F	Breast	S	667	1000
14	49,F	Breast	S	689	1000
15	59,F	Colon	S, 5-FU	806	1250
16	64,M	Colon	S	769	1500
17	77,M	Colon	S	850	1500
18	58,M	Chordoma	S, adriamycin	850	1500

*S = surgical treatment; R = radiation therapy.

TABLE 2.—Toxicity of 5-azacytidine in 18 evaluable patients*

Toxic effect	No. of patients
No toxicity	6
Toxicity	12
Gastrointestinal (total of 12 patients)	
Anorexia	12
Nausea	8
Vomiting	4
Diarrhea	3
Hepatic (total of 5 patients)	
Liver function abnormalities	5
Hepatic coma	3
Hematologic (total of 12 patients)	
Leukopenia	12
Thrombocytopenia	3
Anemia	2
Stomatitis	1
Deaths	5

*Of 22 patients treated with 5-azacytidine, 18 were evaluable for toxicity.

five patients. This consisted of elevations of total bilirubin and/or SGOT levels to at least three times the pretreatment values (baseline bilirubin value, <1.5 mg/100 ml; baseline SGOT value, <120 IU). The three patients who had both abnormal total bilirubin values and abnormal SGOT values died in hepatic coma.

Hepatic coma was diagnosed when a patient manifested lethargy, confusion, icterus, and asterixis with elevated values for bilirubin and SGOT but with normal values for BUN, blood glucose, serum electrolyte concentrations, and serum calcium. All patients in whom hepatic coma developed experienced a rapidly progressing downhill course and died within 10 days of the onset of icterus. Significant hepatic tumor burden had been confirmed in these patients by liver scan and needle biopsy prior to therapy. Hepatic toxicity did not appear to be dose related.

TABLE 3.—Summary of the cases of patients who died of 5-azacytidine toxicity

Patient No.	Age,sex	Tumor	Total dose (mg/m²)	Cause of death
1	71,F	Adenocarcinoma, ovary	276	Gastrointestinal bleeding secondary to thrombocytopenia
3	61,F	Adenocarcinoma, ovary	370	Hepatic coma
4	64,F	Adenocarcinoma, breast	385	Central nervous system bleeding secondary to thrombocytopenia
10	67,M	Adenocarcinoma, colon	513	Hepatic coma
12	63,M	Melanoma	588	Hepatic coma

Twelve patients experienced a dose-related fall in wbc to less than 50% of pretreatment values. The nadir of the wbc occurred on Day 27 (mean) (range, 21–35 days). Wbc counts returned to normal 10 days (mean) thereafter. Four patients experienced significant leukopenia (wbc <2000 cells/mm³); however, none of the patients had fever or signs of systemic infection. Three patients had platelet counts less than 50,000 platelets/mm³ with petechiae. All three patients subsequently died of drug toxicity. Two deaths were directly attributable to thrombocytopenia. Severe anemia (hemoglobin level <5 g/100 ml) occurred in two patients because of marrow hypoplasia. Both patients were pancytopenic and both died (one of thrombocytopenia and the other of hepatic coma).

Five patients died of drug toxicity (table 3). All five patients were more than 60 years of age and had received prior chemotherapy; the 5-azacytidine dosage was moderate, ie, 276–588 mg/m² total dose.

Response to Therapy

Only two of the 18 evaluable patients experienced clinically useful partial remissions (defined as a decrease of more than 50% in the volume of measurable lesions for 1 month). Both responding patients (one with adenocarcinoma of the breast and one with adenocarcinoma of the ovary) received maintenance therapy with weekly doses of 5-azacytidine and remained in remission for 2 and 3 months, respectively. Remissions, when they occurred, were early and dramatic (fig 2).

DISCUSSION

In this study of sc administered 5-azacytidine, leukopenia and thrombocytopenia were dose related and delayed (21–35 days after the start of treatment). In spite of severe leukopenia, infection was not evident in any patient. However, the appearance of thrombocytopenia signaled a serious hemorrhagic diathesis.

Liver function abnormalities were demonstrated in five of our 18 evaluable patients; three of these patients (all with significant hepatic metastatic disease) died of rapidly progressing hepatic coma. In view of the focal hemorrhagic necrosis and fatty metamorphosis reported to occur in the livers of dogs treated with toxic doses of 5-azacytidine,[11] a prospective study to evaluate the effects of this agent on the human liver was undertaken (17). Histologic evaluation of coded percutaneous needle biopsy specimens of liver from eight patients in whom there was no evidence of hepatic metastatic disease revealed no significant differences between pretreatment and posttreatment specimens. Nevertheless, the recent revelation of preferential and prolonged concentration of this potentially hepatotoxic drug in the liver (Troetel, W. M., personal communication) makes it clear that caution should be exercised in treating patients with significant hepatic tumor burden. Specifically, 5-azacytidine therapy is probably contraindicated in patients in whom replacement of liver tissue by tumor is extensive and who

[11] *See* footnote 10.

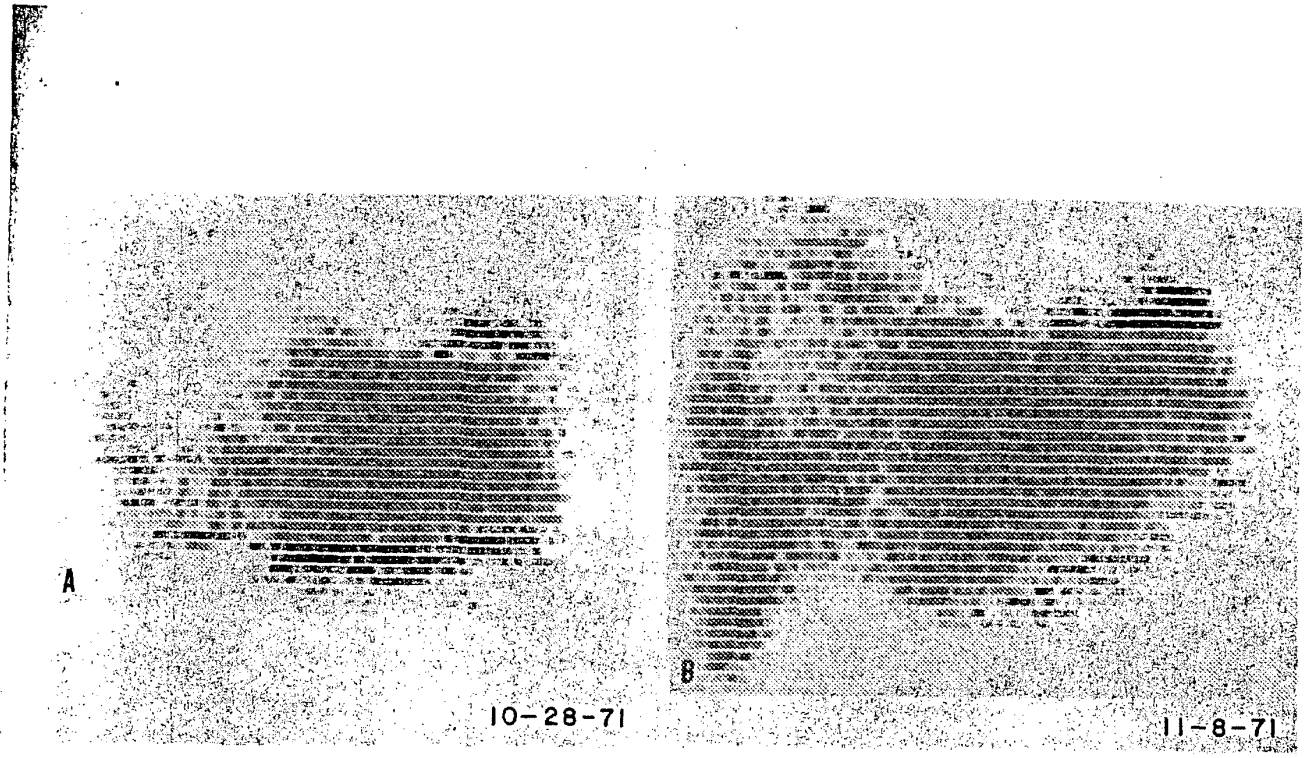

FIGURE 2.—^{99m} Technetium sulfur colloid liver scans of patient No. 2 who had adenocarcinoma of the ovary metastatic to the liver. *A*. Pretreatment scan reveals large filling defects in the superior and lateral inferior aspects of the right lobe of the liver. *B*. Posttreatment scan demonstrates significant improvement in isotope uptake consistent with tumor regression.

have elevated bilirubin values, an SGOT value greater than 120 IU, or a serum albumin value less than 3.0 g/100 ml (17).

Liver function abnormalities and hematologic toxicity are consistent with the findings of other investigators who have administered therapeutic courses of 5-azacytidine (15,18,19). The high incidence of hepatic coma in the present study probably reflects the selection of patients with significant hepatic metastatic disease.

A number of investigators (15,18–21) have been dismayed by the severe dose-limiting gastrointestinal toxicity of 5-azacytidine given iv. Moertel (18, p 652) has stated that "the severity of nausea and vomiting induced by 5-azacytidine seriously compromises any hope of clinical usefulness." However, the sc route produced only mild gastrointestinal toxic effects at doses sufficient to cause significant bone marrow depression.

With respect to antitumor activity, only two patients (one with adenocarcinoma of the breast and one with adenocarcinoma of the ovary) had clinically useful partial remissions. Limited therapeutic activity in solid tumors is consistent with three recent studies reporting objective remissions in two of 21 patients with carcinoma of the breast (20), none of four patients with melanoma (19), and one of 27 patients with cancer of the colon (18). The response rates reported by Weiss (15) (seven of 11 patients with carcinoma of the breast, two of five patients with melanoma, and two of six patients with cancer of the colon) probably reflect the high percentage of patients with soft tissue metastatic disease entered into the study.

Karon et al (21) reported significant antitumor activity of 5-azacytidine in patients with acute childhood leukemia resistant to conventional therapy. Our demonstration of mild gastrointestinal toxicity with significant bone marrow suppression after sc administration provides a useful alternate route of administration of 5-azacytidine to children in whom appropriate sites for venipuncture may be lacking.

CONCLUSIONS

The daily sc administration of 5-azacytidine for 10 days to a total dose of 275–850 mg/m^2 resulted in mild gastrointestinal toxic-

ity and dose-limiting leukopenia. The development of fatal hepatic coma in three patients indicates that care should be exercised in treating patients who have significant hepatic tumor burden. Clinically useful tumor regressions were noted in one of four patients with cancer of the breast, one of four patients with cancer of the ovary, and none of four patients with cancer of the colon. The sc route of administration of 5-azacytidine is a useful alternative to the iv treatment route.

LIST OF COMPOUNDS

Adriamycin: NSC-123127

5-Azacytidine: NSC-102816; CAS reg. No. 320-67-2; *s*-triazin-2(1*H*)-one, 4-amino-1-β-D-ribofuranosyl-

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate

5-FU: NSC-19893; CAS reg. No. 51-21-8; 5-fluorouracil

Hydroxyurea: NSC-32065; CAS reg. No. 127-07-1

Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]-methylamino]benzoyl]-

NSC-45388: CAS reg. No. 4342-03-4; imidazole-4-carboxamide, 5-(3,3-dimethyl-1-triazeno)-; DIC

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Phase II Study of Subcutaneously Administered 5-Azacytidine (NSC-102816) in Patients With Metastatic Malignant Melanoma

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Thirty (30) patients with advanced metastatic malignant melanoma refractory to DTIC (NSC-45388) and a nitrosourea were treated with 5-azacytidine (NSC-102816). 5-Azacytidine was administered subcutaneously at a dosage of 100 mg/m^2/day for 10 days. Twenty-six (26) patients were evaluable for toxicity and response. Major organ toxicities were hematologic, gastrointestinal, and cutaneous; no antitumor activity was noted.

Key words: 5-azacytidine, melanoma, chemotherapy

INTRODUCTION

5-Azacytidine (NSC-102816) is the pyrimidine analog of cytidine with a nitrogen atom substituted for carbon in the number 5 position of the ring. This antimetabolite was originally synthesized by Piskala and Sorm in 1964 (1); subsequently, the nucleoside was isolated from a culture of Streptoverticillum ladakanus (2). The mechanisms of action of 5-azacytidine include competition for uridine kinase (3), inhibition of orotidylic acid decarboxylase (4), and incorporation into RNA (5) and DNA (6). This cancer chemotherapeutic agent has manifest significant antitumor activity in murine L1210 leukemia (7) and human acute myelogenous leukemia (8). The present report details a Phase II evaluation of subcutaneously administered 5-azacytidine in patients with advanced metastatic malignant melanoma.

METHODS

5-Azacytidine was administered to 30 patients with advanced measurable metastatic malignant melanoma. Histologic confirmation of metastatic melanoma of nonocular origin was required for entry into the study. Patients with a life expectancy of less than 3

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months or systemic chemotherapy within 28 days of the initiation of the study were ineligible for treatment. Patients with a hemoglobin value less than 9 g/100 ml, a white blood cell (WBC) count less than 4,000/mm^3, a platelet count less than 100,000/mm^3 or a BUN value greater than 25 mg/100 ml were also ineligible. Patients with a total serum bilirubin greater than 1.5 mg/100 ml, an SGOT greater than 120 IU, or a serum albumin value less than 3 g/100 ml were excluded. The patient population included 18 males and 12 females. The mean age for men was 61 years (range, 34–77 years); the mean age for women was 50 years (range, 25–73 years). All patients had received prior sequential systemic therapy with DTIC (NSC-45388) and a nitrosourea. Fourteen patients had been treated in addition with ICRF-159 (NSC-129943). One female had previously achieved a complete remission lasting 11 months due to therapy with BCNU (NSC-409962) + vincristine. Upon recurrence of metastatic disease, the same patient achieved a partial remission lasting 5 months as a result of systemic chemotherapy with DTIC.

5-Azacytidine was administered at a dosage of 100 mg/m^2/day for 10 consecutive days subcutaneously. Courses of therapy were repeated at 35-day intervals until measurable progression (greater than 25% increase in tumor size) was demonstrated. Palpable dermal, subcutaneous, and lymph node metastases were measured every 5 weeks. Pulmonary parenchymal masses were evaluated with chest X rays at 5-week intervals. Hemoglobin determinations and WBC and platelet counts were performed on days 1, 21, 28, and 35 of each treatment cycle. The BUN, total serum bilirubin, SGOT, alkaline phosphatase, and LDH determinations were performed initially and at 35-day intervals.

5-Azacytidine was supplied as a white lyophilized powder in 100-mg sterile vials. The contents of each vial were suspended in 3 ml of sterile water immediately prior to injection.

This experimental study was approved by the Clinical Investigation Review Committee of the Fox Chase Cancer Center.

RESULTS

Of 30 patients treated with 5-azacytidine, 26 were evaluable for toxicity and response. Three patients died of metastatic melanoma within 21 days of initiation of therapy and were considered unevaluable; 1 patient was lost to follow-up.

Toxicity

Of 26 patients evaluable for toxicity (Table I), 14 developed hematologic side effects. Twelve patients experienced a 50% or greater decrease in WBC count; 3 patients had a greater than 50% decrease in platelet count. The nadirs of leukopenia and thrombocytopenia were both noted on day 28 with recovery by day 35 of each treatment cycle. Overall, there was a statistically significant decrease in WBC and platelet counts on day 28 of the first treatment cycle (Table II). In no case was there evidence of sepsis or hemorrhagic diathesis. No patient developed anemia.

Twenty-five patients experienced nausea during the 10 days of drug administration; 20 experienced emesis, which was controlled with antiemetics. Four patients developed diarrhea. Significant alopecia was noted in 3 patients. Fourteen patients developed pruritis and cutaneous erythema about the sites of subcutaneous injections. There was no evidence of hepatic, pulmonary, neuromuscular, renal, or cardiac toxicity in any patient treated with 5-azacytidine.

TABLE I. Toxicity of 5-Azacytidine in 26 Evaluable Patients*

Type of toxicity	Occurrence/total number of patients
Gastrointestinal	25/26
Nausea	25/26
Emesis	20/26
Diarrhea	4/26
Hematologic	14/26
Leukopenia (≥50%↓)	12/26
Thrombocytopenia (≥50%↓)	3/26
Anemia	0/26
Alopecia	3/26
Cutaneous	14/26

*First course of therapy.

TABLE II. 5-Azacytidine-Induced Hematologic Toxicity in 26 Evaluable Patients*

	Day 1	Day 28	Day 35
White blood cells ($\times 10^3$)†	6.7	4.1 ($p < 0.001$)**	7.4 (p = NS)**
Platelets ($\times 10^3$)†	307	229 ($p < 0.004$)**	356 (p = NS)**

*First course of therapy.
† Mean white blood cell and platelet counts/mm^3.
** Wilcoxon and t tests (NS = $p > 0.05$).

Response

Organ involvement by metastatic melanoma in 26 patients evaluable for response is presented in Table III. No patient experienced objective improvement during treatment with 5-azacytidine; all patients did manifest progressive disease in spite of treatment. Of 26 evaluable patients, 21 experienced progression of disease after one course of therapy. Five patients progressed after 2 courses of 5-azacytidine. Median and mean survivals from initiation of therapy were 3.6 and 4.5+ months, respectively (range, 0.9–12.3+ months).

DISCUSSION

The severe incapacitating emesis resulting from the rapid intravenous administration of 5-azacytidine and the lack of local tissue reaction upon extravasation provided the impetus for a clinical trial with subcutaneously administered 5-azacytidine. The subcutaneous route of administration resulted in mild gastrointestinal toxicity and significant antitumor effect in 1 patient with adenocarcinoma of breast and 1 with adenocarcinoma of ovary (9). In a comparison of intravenous and subcutaneous administration (10), plasma levels of 5-azacytidine were not significantly different 1 hr after injection, and preferential concentration in tumor tissue was demonstrated regardless of the route

TABLE III. Organ Involvement by Melanoma in 26 Evaluable Patients

Patient	Skin	Subcutaneous tissue	Lymph nodes	Lung	Liver	Bone	Brain
1	X	X	X				
2	X	X	X		X		
3		X	X	X			
4	X			X			
5		X	X				
6		X	X	X			
7			X		X		
8		X	X				
9	X	X		X			
10		X	X	X			
11		X	X	X			X
12		X	X	X		X	X
13	X	X	X	X		X	
14		X	X	X			
15	X	X	X	X			
16		X	X	X			
17				X		X	X
18		X		X		X	
19		X	X	X	X	X	
20		X				X	
21	X	X		X			
22		X		X			
23		X	X		X		X
24	X	X					
25	X	X	X	X	X	X	
26		X	X				

of administration. Subcutaneous administration of 5-azacytidine was therefore deemed a useful alternative to the standard intravenous route (9).

Weiss et al. (11) reported 3 objective remissions in 5 patients with metastatic malignant melanoma who were treated with intravenous 5-azacytidine. The dearth of active agents for the treatment of metastatic malignant melanoma led us to the present Phase II trial in patients with advanced melanoma refractory to DTIC and a nitrosourea.

The spectrum of toxicity manifest in the present trial is consistent with the toxicity data reported in earlier studies of intravenously administered 5-azacytidine (8). The high incidence of nausea and emesis in the present study is likely a dose-related phenomenon; the prior clinical trial of subcutaneously administered 5-azacytidine utilized a mean dosage of 50 mg/m^2/day for 10 days (9). It therefore appears that the subcutaneous route of administration does not result in a diminished incidence of gastrointestinal toxicity.

No objective remissions were produced in 26 previously treated patients with metastatic melanoma who received 5-azacytidine in therapeutic dosage. In fact, there was no suggestion of disease stabilization. The 5 patients who received 2 courses of 5-azacytidine had slowly progressive disease prior to therapy which continued unabated during treatment with 5-azacytidine. Although all 26 patients had advanced disease and significant tumor burdens, 25 had measurable dermal, subcutaneous, or nodal metastases and

were, therefore, reasonable candidates for response. In addition, a median survival of 3.6 months does not reflect a treatment population composed of terminally ill patients.

An objective response rate of 0/26 has a less than 7% (0.90^n, n=26) chance of occurring with an agent having a true response rate (TRR) $\geqslant$ 10%. Since we consider cancer chemotherapeutic agents with a less than 50% probability of having a TRR $\geqslant$ 10% to be inactive in the treatment of patients with metastatic melanoma (12), 5-azacytidine is deemed inactive in this disease.

CONCLUSIONS

5-Azacytidine administered subcutaneously at a dosage of 100 mg/m^2/day for 10 consecutive days is ineffective in the treatment of patients with advanced metastatic melanoma who have received prior chemotherapy. This lack of antitumor activity does not suggest that 5-azacytidine be afforded a trial in the treatment of patients with malignant melanoma at an earlier stage in their metastatic disease.

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Cell Cycle Phase Specificity of Antitumor Agents[1]

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SUMMARY

The sensitivity to drugs of synchronous and asynchronous populations of DON cells was studied. Agents that were cytotoxic at a specific phase of the cell cycle gave dose-survival curves that decreased to a constant saturation value. DNA synthesis inhibitors such as 1-β-D-arabinofuranosylcytosine, 5-azacytidine, 5-hydroxy-2-formylpyridinethiosemicarbazone (NSC 107392), sodium camptothecin (NSC 100880), 5-fluorodeoxyuridine, and pseudourea (NSC 56054) were most cytotoxic to cells in the S phase. However, the DNA synthesis inhibitor, neocarzinostatin, was most cytotoxic to cells in the G_1 phase. The protein synthesis inhibitors, pactamycin and sparsomycin, were also most cytotoxic to cells in the S phase. Cells in the G_1-S border region were most sensitive to the RNA synthesis inhibitors, actinomycin D and nogalamycin, and to the alkylating agents, 1,3-bis(2-chloroethyl)-1-nitrosourea, and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea. Streptozotocin and tubercidin, which markedly inhibit the synthesis of DNA, RNA, and protein, were cytotoxic to cells in all phases of the cell cycle. 5-Fluorouracil was also not phase specific. Cells in mitosis and in the G_1 phase were most sensitive to chlorambucil, L-phenylalanine mustard, and ellipticine.

INTRODUCTION

Bruce *et al.* (12) have classified a number of chemotherapeutic agents into 3 groups. The 1st group, consisting of γ-radiation and nitrogen mustard, killed both proliferative and resting cells in all portions of the generation cycle. In contrast, the agents in the 3rd group killed only proliferative cells in all phases of the cell cycle. Agents belonging to both of these groups gave exponential curves, when survival was measured at different drug concentrations. The 2nd group of agents gave dose-survival curves that decreased to a constant saturation value at high doses, indicating that they killed cells in 1 portion of the cell cycle; *i.e.*, these agents were phase specific.

The variation in the sensitivity of cultured mammalian cells through the division cycle to different agents has been reported. The recent paper by Mauro and Madoc-Jones (33) summarizes the results obtained with a large number of agents. Such studies are useful for 2 reasons: (*a*) They may enable us better to understand the parameters of the cell cycle by correlating the biochemical effect of the drug to its lethal effect. For example, many drugs are maximally toxic to cells at the G_1-S boundary, and a correlation between the lethal and biochemical effects of the drug may help to delineate the biochemical reaction that triggers the cell across the G_1-S boundary. (*b*) They may help in designing combinations of drugs on a rational basis. Thus, 2 drugs affecting the same phase of the cell cycle would not give additive effects, if combined.

Our studies were intended to determine the phase specificity of several clinically active agents and of agents with known biochemical activity. Parts of this study were reported previously (7).

MATERIALS AND METHODS

Cell Culture. DON cells. from a Chinese hamster fibroblast line (ATCC CCL16), were grown at 37° in McCoy's 5A medium modified by the addition of lactalbumin hydrolysate (0.8 g/liter) and fetal calf serum (200 ml/liter). The medium was obtained from the Grand Island Biological Company, Grand Island, N.Y. The cells were grown in 8-oz prescription bottles planted with about 2×10^6 cells in 25 ml of medium and were maintained in logarithmic growth by subculture every 2 days. With renewal of the medium every 2 days, an 8-oz bottle could support logarithmic cell growth, with a generation time of 10 to 12 hr, until there were about 10^8 cells/bottle. For subculturing, the cell monolayer was detached from glass by treatment with a 0.1% trypsin solution; the cells were then dispersed in medium, and an aliquot was planted in bottles.

Synchronous DON Cells. A modification of the method of Stubblefield *et al.* (42) was used to obtain a population of mitotic cells. Logarithmically growing cells were planted in roller bottles (11 x 28.5 cm; 840-sq cm growth surface; Bellco Glass, Inc., Vineland, N. J.) at about 10^7 cells in 200 ml of medium. The bottles were gassed with a 5% CO_2:95% air mixture and were rotated for 2 days on a roller apparatus (Bellco) at 0.6 rpm. After 2 days of incubation, Colcemid (demecolcine; Ciba Pharmaceutical Company, Summit, N. J.) was added to the medium to give 0.06 μg/ml, and the bottles were incubated for 3 more hr. The medium was then poured off, and we selectively removed the mitotic cells by rolling the bottles with 40 ml of trypsin (at 4°; 0.125 mg/ml) and 1 ml of $NaHCO_3$ (7.5%). The mitotic cells were centrifuged and resuspended in medium at 4°, and about 10^6 cells were planted in 3-oz bottles containing medium at 37°. The mitotic cells constituted between 85 and 95% of the harvested cells.

Determination of Percentage of Cell Survival after Exposure of DON Cells to Drug. Cells growing in synchrony were

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exposed to drug for 1 hr at different times after the planting of mitotic cells to expose cells in different parts of the cell cycle. Asynchronous cells were also exposed to the drug for 1 hr.

After exposure to drug, the supernatant medium was poured off, and the cells were detached with trypsin and resuspended in medium at 37°. When it was suspected that the drug might also detach cells, the supernatant medium was also centrifuged, and these cells were added to the total cell pool. The cells were diluted in medium at 37°, and 2 ml of cells were planted in plastic Petri plates (Linbro Chemical Co., New Haven, Conn.) to give 10 to 100 colonies per plate. Twelve plates were planted for each sample. After incubation for 7 to 8 days in an atmosphere of 8% CO_2:92% air at 37°, the medium was poured off, and the colonies were stained with 0.2% methylene blue in 70% ethanol. The colonies were counted with a Quebec colony counter (Spencer Lens Co., Buffalo, N. Y.). The plating efficiencies were about 50% for synchronous cells and 70% for asynchronous cells. For calculation of the percentage of survivals, the control (no drug treatment) samples were normalized to 100% survival. In these experiments, the coefficient of variation in determining cell survival was about 15%, the coefficient of variation being the standard deviation expressed as a percentage of the mean. Thus, if the percentages of survival of 2 different samples were 50 and 30%, respectively, then they would be statistically significantly different at the 95% confidence level. Almost all experiments were repeated, and the results were found to be reproducible; in duplicate determinations, the individual values were within 10% of the mean value.

Determination of Macromolecule Synthesis. Asynchronous DON cells were planted at 10^6 cells/3-oz bottle in 10 ml of medium. After overnight incubation, the cells were refed with fresh medium, and drug and labeled precursors were added for 1 hr. To stop uptake of radioactivity, cells were detached with 0.1% trypsin containing unlabeled precursors (100 μg/ml), and the cells were suspended in 0.9% NaCl solution. One aliquot was counted in the Coulter counter to give the cell number, while another (1-ml) aliquot of cells was filtered through 0.45-μ Millipore filters. The filter was washed 4 times with cold 10% trichloroacetic acid and once with ethanol. The filter was then incubated with 0.5 ml of 0.5 N perchloric acid at 70° for 20 min. Diotol (15 ml) was added, and the filter was counted in a scintillation counter.

Autoradiography and Mitotic Index Determination. The slides were prepared for autoradiography and mitotic index determinations, as previously described (28).

Drug Samples. The drugs with the NSC numbers were obtained from Chemotherpy, National Cancer Institute, Bethesda, Md. The other drugs (ara-C,[2] 5-azaCR, Tu, streptozotocin, nogalamycin, pactamycin, sparsomycin, and cycloheximide) were developed by The Upjohn Company, Kalamazoo, Mich. The solubilities and structures of the drugs are as follows. FUdR, neocarzinostatin (NSC 69856; Ref. 32), thio-TEPA (NSC 6396), cycloheximide, ara-C, 5-azaCR, sodium camptothecin (alkaloid; NSC 100880; Ref. 20), and streptozotocin (22) were soluble in H_2O, Tu, and sparsomycin (45) were soluble in hot H_2O. FU was soluble at 20 mg/ml in 7.5% $NaHCO_3$. Nogalamycin (46) and actinomycin D were dissolved in acetone at 1 mg/ml. 5-Hydroxy-2-formylpyridinethiosemicarbazone (NSC 107392), pactamycin (44), BCNU (NSC 409962), CCNU (NSC 79037), and chlorambucil [4{*p*-[bis(2-chloroethyl)amino]phenyl}butyric acid; NSC 3088] were dissolved in ethanol at 5 to 10 mg/ml. Pseudourea (NSC 56054; Ref. 13) was dissolved at 10 mg/ml dimethyl sulfoxide. Ellipticine (NSC 71795; Ref. 20) and L-phenylalanine mustard [3-(*p*-[bis(2-chloroethyl)amino]phenyl-L-alanine; NSC 8806] were dissolved at 10 mg/ml in 0.1 N HCl.

When we used solvents for solutions of drugs, we attempted to prepare a 10 mg/ml solution. Thus, it was possible to dilute out the solvent in the process of diluting the drug to the desired concentration. The drug concentrations used to determine sensitivity of different phases were chosen to show a well-defined age response. In many cases, 2 or 3 different levels of the drug were used (see Charts 2, 4, etc.), but the sensitivity of different phases to the drug pertains only to the concentrations used and is not necessarily true at much higher drug concentrations.

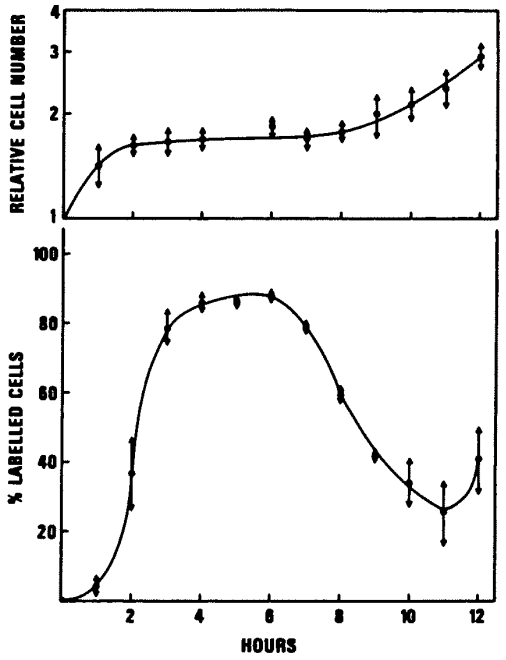

Chart 1. Cell cycle of synchronized DON cells. Mitotic cells, planted in 3-oz bottles, were pulsed with TdR-^{3}H (1 μCi/ml, 2 Ci/mmole) for 10 min at different times after planting. The cells were then harvested, and cell counts, mitotic index, and percentage of ^{3}H-labeled cells were determined. The curves show the mean ± average deviation from the mean of 4 separate experiments.

[2] The abbreviations used are: ara-C, 1-β-D-arabinofuranosylcytosine (Cytosar); 5-azaCR, 5-azacytidine; Tu, tubercidin; FUdR, 5-flurodeoxyuridine; thio-TEPA, tris(1-aziridinyl)phosphine sulfide; FU, 5-fluorouracil; NSC 107392, 5-hydroxy-2-formylpyridinethiosemicarbazone; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; pseudourea, 2,2'-(9,10-anthrylenedimethylene)bis(2-thiopseudourea)dihydrochloride dihydrate; TdR, thymidine.

Table 1
Inhibition of macromolecule synthesis in DON cells by various agents

Agent	Dose (μg/ml)	Inhibition[a] (%) DNA	RNA	Protein
ara-C[b]	100	92	10	24
ara-C	1000	96	24	17
5-AzaCR[c]	100	91	87	95
NSC 107392[b]	2	85	14	1
Sodium camptothecin[b]	1.85	60	53	0
Sodium camptothecin	9.2	77	64	
Tu[d]	2	84	86	88
Streptozotocin[e]	1000	37	0	19
Streptozotocin	5000	75	68	79
FU[c]	100	37	84	0
FUdR[c]	100	40	64	41
Neocarzinostatin[c]	5	44	−20	−9
Neocarzinostatin	50	60	−30	−31
Actinomycin[b]	1	7	87	0
Nogalamycin[b]	2	40	74	11
Pactamycin[b]	1	79	46	98
Pactamycin	100	80	91	99
Sparsomycin[b]	100	80	35	98
Pseudourea[e]	1.1	52.6	35.1	25
Pseudourea	10	81	90	40.4

[a] In all cases, the DON cells were incubated with radioactive precursors for 1 hr. TdR-^{3}H, uridine-5-^{3}H, and DL-valine-^{14}C were added to give 5 μCi/2 μg/ml, 5 μCi/5 μg/ml, and 0.1 μCi/23 μg/ml, respectively. The specific activities of control (no drug) samples of DNA, RNA, and protein were (per 10^6 cells): 13.5×10^5, 20×10^3, and 16.1×10^3 cpm, respectively. All samples were in duplicate.

[b] DON cells were exposed to agent and radioactive precursor for 1 hr.

[c] Cells were exposed to agent for 5 hr prior to addition of radioactive precursor for 1 hr.

[d] Cells were exposed to Tu for 2 hr prior to addition of labeled precursor for 1 hr.

[e] Cells were exposed to agent for 1 hr prior to addition of labeled metabolities. The agents in Group *b* rapidly inhibited macromolecule synthesis, while the other agents needed a longer period of contact with cells. Neocarzinostatin stimulated the incorporation of precursors into RNA and protein.

RESULTS

Cell Cycle of Synchronous DON Cells. The results obtained when mitotic DON cells were planted as a monolayer are shown in Chart 1. The mitotic index in different experiments ranged from 0.85 to 0.95. Mitosis was completed within 1 hr, as shown by decrease in mitotic index from about 0.9 to < 0.05. Cell division was not complete until 2 hr after mitotic cells were planted, and the relative cell number increased from 1 to 1.7 during this period. Within 3 hr after planting, about 80% of the cells had entered S phase (labeled by TdR-^{3}H pulse), and they remained in S for 7 hr. After 7 hr, cells started entering G_2 with some loss of synchrony. Cell division commenced about 9 hr from the time of planting of mitotic cells. Therefore, under these conditions, the approximate time taken for the different phases (after planting of mitotic cells) is from 0 to 2 hr to complete mitosis and G_1, from 2 to 8 hr for S, and from 8 to 11 hr for G_2 and mitosis.

Inhibition of Macromolecule Synthesis by Several of the Agents. For convenience in presentation, the agents have been subdivided into classes on the basis of their inhibition of macromolecule synthesis and known sites of action. This division does not, however, imply that a given agent has only 1 biochemical mode of action. The subdivision merely indicates that the agent in question markedly inhibits the synthesis of a particular macromolecule. Thus both ara-C and Tu inhibit DNA synthesis (Table 1) and are classified as DNA synthesis inhibitors. However, only ara-C specifically inhibits DNA synthesis, while Tu inhibits almost equally the synthesis of DNA, RNA, and protein. The sites of action of many of these agents have recently been compiled by Livingston and Carter (29). Other pertinent publications on their sites of action are listed below after each compound.

The inhibition of macromolecule synthesis by several of the agents is shown in Table 1. ara-C (14, 19), 5-azaCR (28), NSC 107392 (11), sodium camptothecin (23), Tu (1), streptozotocin (5), FU (21, 31), FUdR (21), and neocarzinostatin (27, 34) have been classified in the following section as DNA synthesis inhibitors. Among them only ara-C, NSC 107392, and neocarzinostatin markedly inhibited DNA synthesis without simultaneous marked inhibiton of RNA and protein synthesis. The other agents in this group markedly inhibited DNA, RNA, and/or protein synthesis.

Actinomycin D (38) and nogalamycin (8) have been classified as RNA synthesis inhibitors. Actinomycin D was a much more specific inhibitor of RNA synthesis than was nagalamycin.

Pactamycin (Ref. 18, pp. 169–173) and sparsomycin (Ref.

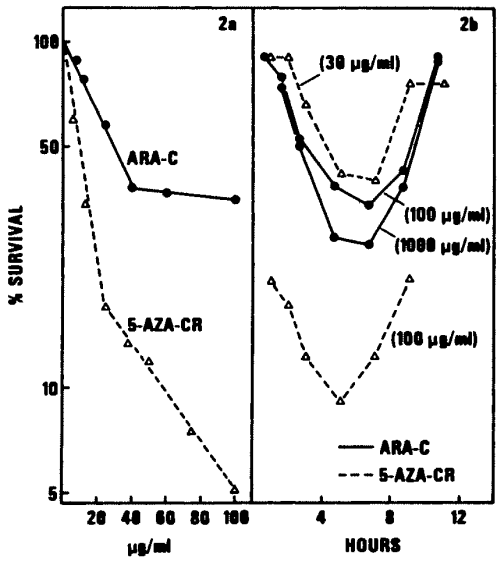

Chart 2. Sensitivity of DON cells to 5-azaCR and ara-C. In *a*, asynchronous cells were exposed to different levels of drugs for 2 hr. The drug was removed, and the cells were diluted and planted. Colonies were counted after 7 to 8 days of incubation. In *b*, mitotic cells were exposed to the drugs for 1-hr periods after planting. The drugs were removed after 1 hr, and the cells were diluted and planted in 12 plates for each sample. Colonies were counted after 7 to 8 days of incubation. The coefficient of variation in percentage of survival was about 15%. The times shown are halfway points in the period of exposure, *i.e.*, cells exposed at 1 hr after planting and harvested at 2 hr are shown at 1.5 hr.

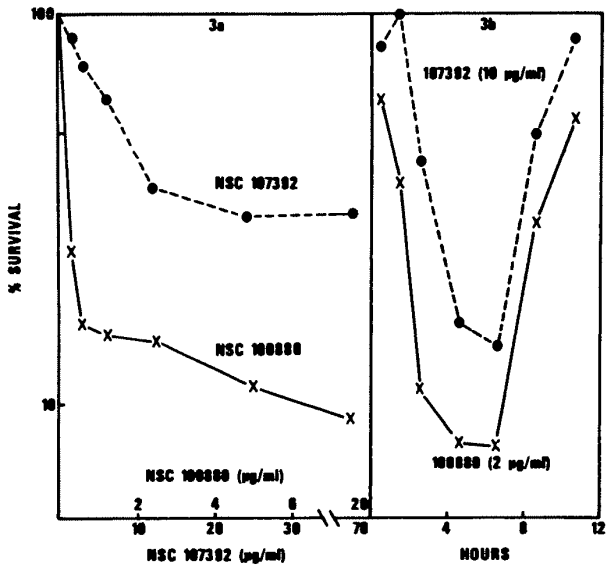

Chart 3. Sensitivity of asynchronous (*a*) and synchronous (*b*) DON cells to NSC 107392 and sodium camptothecin (NSC 100880). Protocol same as Chart 2, except drug exposure was for 1 hr.

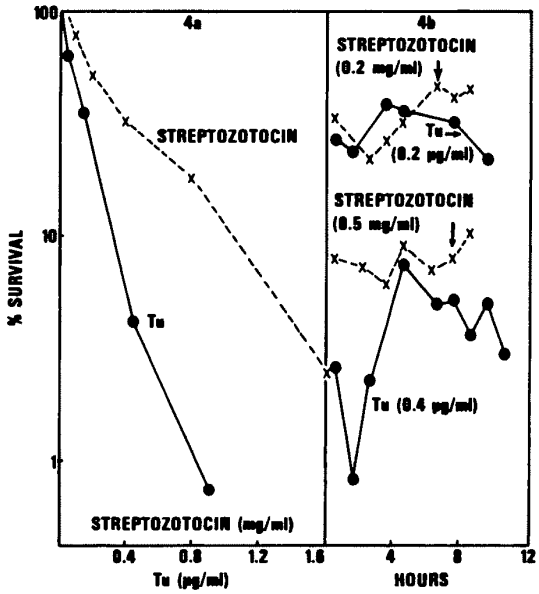

Chart 4. Sensitivity of asynchronous (*a*) and synchronous (*b*) DON cells to streptozotocin and Tu. Protocol same as in Chart 3.

18, pp. 410–415) almost completely inhibited protein synthesis, although DNA and RNA synthesis were also markedly inhibited.

BCNU (Ref. 29, pp. 360–365), CCNU, chlorambucil (Ref. 29, pp. 81–98), thio-TEPA, and phenylalanine mustard (Ref. 29, pp. 99–111), have been classified as alkylating agents.

Response to DNA Synthesis Inhibitors. The dose-survival curves of asynchronous cells exposed to ara-C (Chart 2*a*) decreased to a constant saturation value at high doses, indicating that ara-C is cytotoxic to a specific phase or phases of the cell cycle. Results obtained with synchronous cells (Chart 2*b*) showed that, at low levels, both ara-C and 5-azaCR were specifically cytotoxic to S-phase cells. High levels of 5-azaCR (100 μg/ml) were lethal to cells in G_1, S, G_2, and M, although cells in S phase were still most sensitive. However, ara-C at high level (1 mg/ml) was still lethal only to cells in S.

The dose-survival curve for NSC 107392 (Chart 3*a*) decreased to a constant saturation value, indicating that this

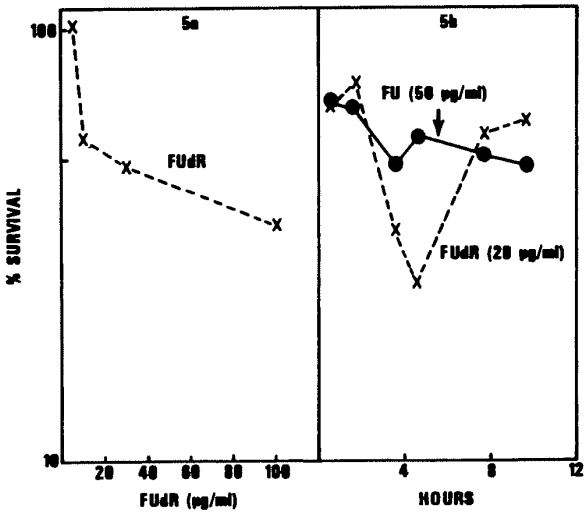

Chart 5. Sensitivity of asynchronous (*a*) and synchronous (*b*) DON cells to FU and FUdR. Protocol same as in Chart 3.

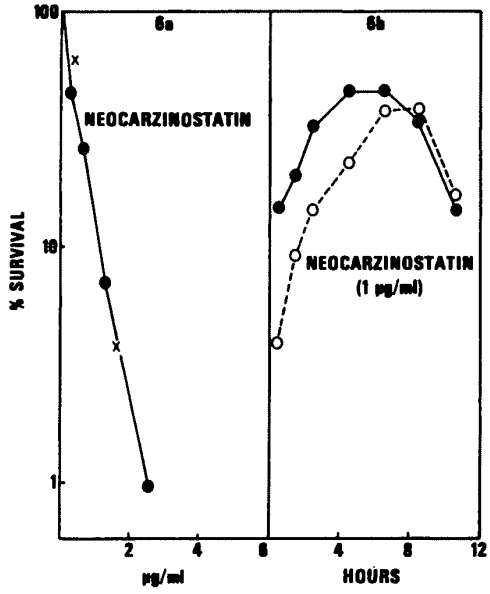

Chart 6. Sensitivity of asynchronous (*a*) and synchronous (*b*) DON cells to neocarzinostatin. Protocol same as in Chart 3. The results of 2 separate experiments are shown.

drug is cytotoxic to a specific phase or phases of the cell cycle. The dose-survival curve for sodium camptothecin (Chart 3*a*) suggests the presence of cell populations with varying sensitivities to the drug. The results with synchronous cells (Chart 3*b*) show that both of these agents are most cytotoxic to cells in the S phase. The percentage ot cell kill obtained in different phases with sodium camptothecin, 10 μg/ml, was approximately the same as the percentage of cell kill at 2 μg/ml. Although both DNA and RNA synthesis were markedly inhibited by sodium camptothecin, 10 μg/ml (Table 1), the drug was still maximally lethal to cells in S. However, unlike NSC 107392, cells in M, G_1, and G_2 were killed by sodium camptothecin.

The results with asynchronous cells exposed to streptozotocin are shown in Chart 4*a*. The results with synchronous cultures (Chart 4*b*) showed that cells in all phases of the cell cycle were equally sensitive to the drug. This becomes particularly evident at the higher concentration (0.5 mg/ml) of streptozotocin.

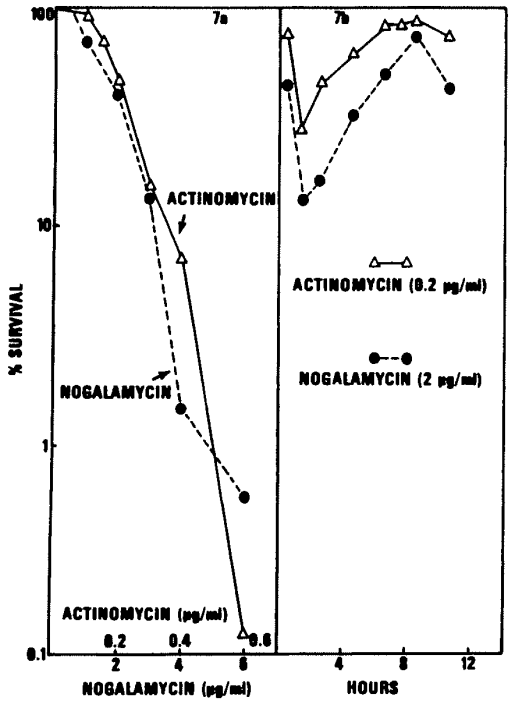

Chart 7. Sensitivity of asynchronous (*a*) and synchronous (*b*) DON cells to actinomycin D and nogalamycin. Protocol same as in Chart 3.

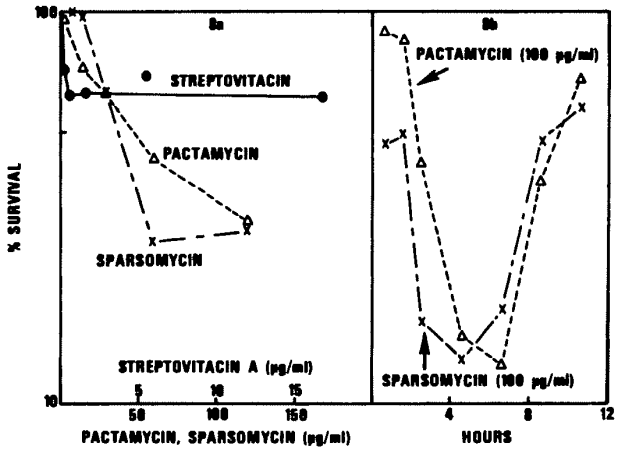

Chart 8. Sensitivity of asynchronous (*a*) and synchronous (*b*) DON cells to streptovitacin, pactamycin, and sparsomycin. Protocol same as in Chart 3.

Table 2

Correlation of cell kill with percentage of inhibition of protein synthesis before and after washing off of the drug

Drug	Dose (μg/ml)	Cell kill[a] (%) AT 1 hr	Cell kill[a] (%) At 4 hr	Inhibition of protein synthesis (%) 1 hr[b] (before washing)	1 hr[c] (after washing)	4 hr[d] (after washing)
Pactamycin	0.1	17.2	45.4	99.9	19	52.5
Pactamycin	1.0	24	64.9	99.97	54.5	62
Pactamycin	10	27.5	80.5	99.9	91.9	91.2
Puromycin	100	23.2	64.8	99.6	0	38.5
Cycloheximide	100	21.2	57.5	99.9	20	78

[a] After 1- or 4-hr exposure to the drug, the cells were washed, diluted, and planted to determine the percentage of surviving cells.

[b] The cells were exposed to the drugs for 1 hr. Immediately after drug exposure, the cells were given a 20-min pulse with DL-valine-1-^{14}C (1 μCi/23 μg/ml medium) to determine the percentage of inhibition of protein synthesis before washing. A similar percentage of inhibition of protein synthesis (before washing) was obtained when cells were exposed to the drug for 4 hr.

[c] For determination of the percentage of inhibition of protein synthesis after washing, the cells were washed after 1 hr of drug exposure, were allowed a 1-hr recovery period, and then were given a 20-min pulse of DL-valine-1-^{14}C. Control cells incorporated 10^4 cpm/2 × 10^6 cells into the acid-insoluble fraction.

[d] Same as above, except cells were exposed to drug for 4 hr.

In synchronous cultures, Tu at low levels (0.2 μg/ml) was approximately equally cytotoxic to cells in all phases of the cell cycle (Chart 4*b*). However, at high levels (0.4 μg/ml), cells at the G_1-S boundary region seemed to be more sensitive to Tu than were the cells in G_1 or S.

FU was equally cytotoxic to cells in all phases of the cell cycle (Chart 5*b*). With asynchronous cells, the dose-survival curve for FUdR had a shoulder preceding the exponential component of the curve, since there was no cell kill with FUdR at 5 μg/ml or less. The exponential component of the dose-survival curve had 2 different slopes, indicating cell populations with different sensitivities to the drug (Chart 5*a*). This was reflected in the greater sensitivity of cells in the S phase to FUdR.

The results obtained in 2 different experiments with neocarzinostatin are shown in Chart 6. The difference between the 2 experiments with neocarzinostatin could be due to the variation in the neocarzinostatin preparation. With synchronous cells, both experiments (in spite of the difference in percentage of survival values) show that the drug was maximally cytotoxic to cells in M and G_1 phases. The sensitivity decreased as the cells progressed through S and increased again as the cells entered G_2 and M.

RNA Synthesis Inhibitors. The dose-survival curves with asynchronous cells (Chart 7*a*) showed that actinomycin D, 0.1 μg/ml, and nogalamycin, 1 μg/ml, did not kill any cells, indicating the presence of a shoulder region preceding the exponential component. The results with synchronous cells (Chart 7*b*) indicated that cells in the G_1-S boundary region were most sensitive to the agent. These results confirm those of Elkind *et al.* (15) and Mauro and Madoc-Jones (33).

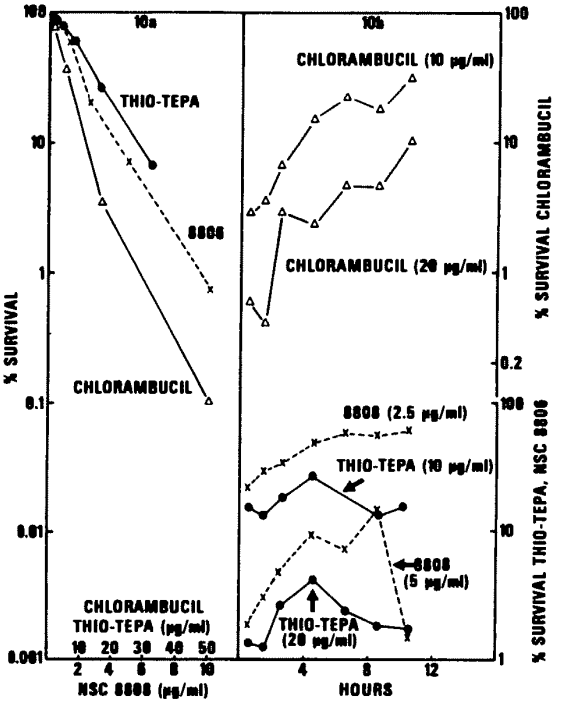

Chart 10. Sensitivity of asynchronous (*a*) and synchronous (*b*) DON cells to chlorambucil, thio-TEPA, and L-phenylalanine mustard. Protocol same as in Chart 3.

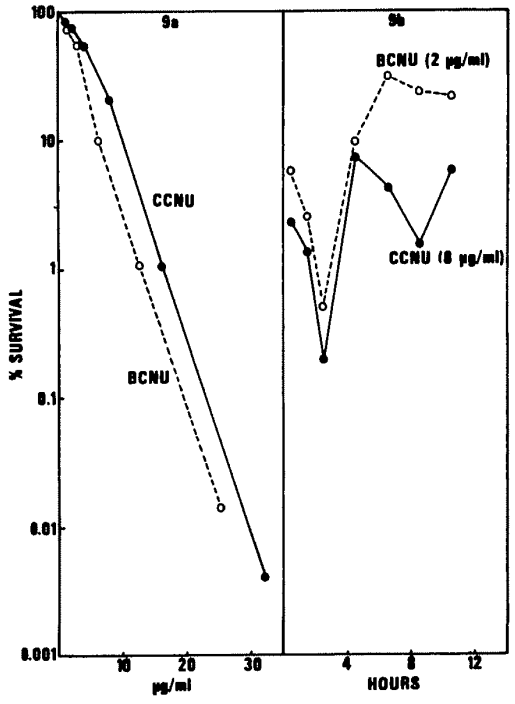

Chart 9. Sensitivity of asynchronous (*a*) and synchronous (*b*) DON cells to BCNU and CCNU. Protocol same as in Chart 3.

Protein Synthesis Inhibitors. The dose-survival curves of asynchronous cells exposed to pactamycin, sparsomycin, and streptovitacin A are shown in Chart 8*a*. Only sparsomycin showed a shoulder region prior to the exponential component of the curve. Streptovitacin A and sparsomycin curves reached saturation values for percentage of survival at high doses, indicating that these agents are phase specific. The results obtained with synchronous cells are given in Chart 8*b*. Cells in S phase were most sensitive to pactamycin and sparsomycin. Cells in S phase have been shown to be sensitive to streptovitacin A by Mauro and Madoc-Jones (33).

The dose (in μmoles/ml) for 50% cell kill after 1 hr of exposure to several protein synthesis inhibitors was: streptovitacin A, 0.0018; pactamycin, 0.025; sparsomycin, 0.095; puromycin, > 0.2; and cycloheximide, > 0.7. These results indicate that, except for streptovitacin A, the protein synthesis inhibitors tested (pactamycin, puromycin, cycloheximide, and sparsomycin) were relatively ineffective in killing cells after 1 hr of exposure. The results in Table 2 may indicate a possible explanation of this effect. Cells exposed to protein synthesis inhibitors for 1 hr have greater than 99% of their protein synthesis inhibited, and yet only 25% of the cells were killed. This was due to the fact that when the agents were removed by washing after 1 hr of exposure, the cells recovered their protein synthesizing ability. This was not true when high levels of pactamycin were used, possibly indicating that all of the pactamycin was not removed by washing. However, after 4

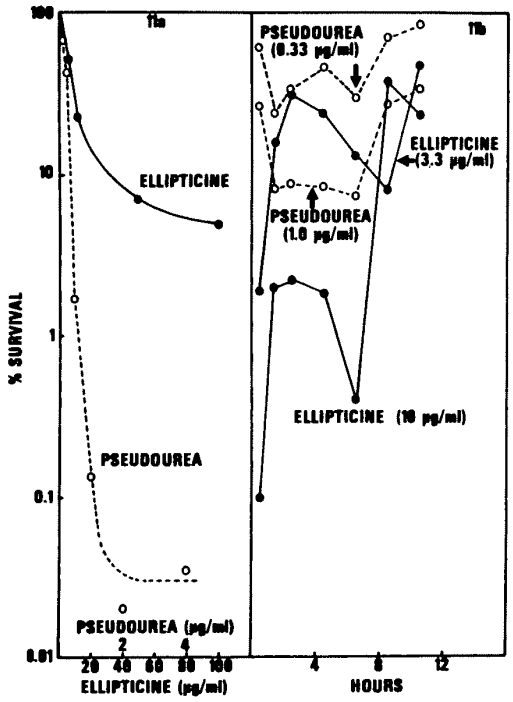

Chart 11. Sensitivity of asynchronous (*a*) and synchronous (*b*) DON cells to ellipticine and pseudourea. Protocol same as in Chart 3.

hr of exposure to the drug, the protein synthesis machinery had been severely damaged so that the cell did not completely recover its synthetic capacity. Therefore, the percentage of inhibition of protein synthesis after the drug had been washed off compared favorably with the percentage of cell kill.

Alkylating Agents. BCNU and CCNU (Chart 9*b*) were most cytotoxic to cells in G_1-S border or early S. With both BCNU and CCNU, the sensitivity of cells decreased as the cells progressed into S. However, with CCNU and to a lesser extent with BCNU, the cell sensitivity increased again as the cells entered late S or G_2.

Thio-TEPA, phenylalanine mustard, and chlorambucil were most cytotoxic to cells in the M and G_1 phase (Chart 10*b*). At the higher concentration of phenylalanine mustard and with thio-TEPA, cells in G_2 were as sensitive as the G_1 cells.

Miscellaneous Agents. The results with pseudourea and ellipticine, 2 agents with unknown sites of action, are shown in Chart 11. Pseuodourea was most cytotoxic to cells in S, while ellipticine was most cytotoxic to cells in M and G_1. As the cells progressed through S, their sensitivity to ellipticine decreased, but ellipticine toxicity increased again as the cells passed through late S or early G_2. The greater sensitivity to ellipticine of cells in M and G_1 as compared with cells in S or G_2 was seen at drug doses ranging from 0.36 μg/ml (not shown in chart) to 10 μg/ml.

The inhibition of macromolecule synthesis by pseudourea is shown in Table 1. Pseudourea inhibited DNA synthesis more than RNA or protein synthesis at the lower concentrations, while at 10 μg/ml of the drug, DNA and RNA syntheses were equally inhibited.

DISCUSSION

The selective detachment method of Terasima and Tolmach (43) for obtaining mitotic cells does not subject the cells to any perturbations and is therefore the ideal method for synchronizing cells. However, the low yield of mitotic cells forced us to use the present method of selectively removing mitotic cells from a culture treated with Colcemid. Stubblefield *et al.* (42) reported that the cells suffered no lasting effects from a 2-hr Colcemid (0.06 μg/ml) treatment. However, Kato and Yosida (24) have shown that chromosomal nondisjunction occurred frequently after Colcemid treatment and that the plating efficiency of synchronized cells was lower than that of untreated control cells. We found that synchronized cells had about 70% of the plating efficiency of asynchronous cells. Also, when an asynchronous population of DON cells was exposed to Colcemid (0.06 μg/ml) for 4 hr, only about 80% of the cells survived. These results indicate that our synchronized population may contain a certain percentage of dead or dying cells. However, the measurement of reproductive cell survival (or colony-forming ability) after treatment with cytotoxic agents is not affected by the presence of dead cells in the culture at the time of drug treatment. Although 20% of the cells had lost their reproductive potential, they still were able to synthesize DNA, since almost 90% of the cells were labeled with TdR-^{3}H pulse (Chart 1).

The index of synchrony (F) was determined by the method of Blumenthal and Zahler (9) to be 0.59. This compared favorably with the F values of 0.6 of Kim and Stambuck (26) and the calculated F value of 0.65 for the synchronized cultures of Pfeifer and Tolmach (35).

During the 1st cell division, the relative cell number increased from 1 to 1.7 (instead of the expected 2) during a 2-hr period. During the 1st hr, mitotic division was completed, and the mitotic index decreased from 0.9 to < 0.05. This result might indicate that some of the cells completed mitotic division but either did not complete cell division or did not separate after cytokinesis and were counted as 1 cell in the Coulter counter. Pfeiffer and Tolmach (35) found that their relative cell number increased from 1 to 1.8.

The cells stayed in S till about 7 hr, and then they began entering G_2 with some loss of synchrony. The loss of synchrony increased as the cells progressed through the cell cycle from G_1 to S to G_2. This is indicated by the steeper slope of the percentage-labeled cell curve as the cells go from G_1 to S, compared with the slope of the curve as the cells go from S to G_2. Such loss of synchrony is due to the variation in the intermitotic times of individual cells, as shown by Sisken (40).

In our experiments, the cells were exposed to drug usually for 1 hr. Such a short period of exposure was chosen for 2 reasons: (*a*) the G_1 and G_2 phases of DON cells are of less than 2-hr duration. Therefore, in order to expose cells in these phases of the cell cycle, it was necessary to use a short exposure period. (*b*) Many drugs have a short plasma half-life, such that usually the cells *in vivo* are exposed to drug for a short time. For example, ara-C (10), pactamycin (6), 5-azaCR (37), actinomycin D (36), and streptozotocin (39) have plasma half-lives of about 20 min, < 5 min, < 5 min, < 5 min, and 5

min, respectively. Therefore, cytotoxicity of a drug determined after 1 hr of exposure might correlate better with the *in vivo* effects of the drug than might the usual method of cytotoxicity determination, wherein the cells are exposed to the drug for 2 to 3 days.

Mauro and Madoc-Jones (33) suggested that most of the drugs studied gave dose-survival curves in which a shoulder region preceded the exponential portion of the curve. The presence of a shoulder region suggests that the cells accumulate damage which ultimately leads to a lethal effect and that survivors have sublethal damage which is repaired (16). Of all the agents reported here, only FuDR, nogalamycin, actinomycin D, and sparsomycin gave dose-survival curves in which a shoulder region was obviously present.

At the present state of our knowledge, it may not be possible to predict the most susceptible phase from a knowledge of the biochemical site of action. However, attempts have been made to explain the sensitivity of different phases on the basis of the site of action of the drug; this is discussed below.

(*a*) For a valid correlation, exposure to a given drug should be completely terminated by washing the cells to remove the drug. However, nucleosides such as Tu, 5-azaCR, FU, etc., are converted to their respective nucleotides inside the cell and may be held in the intracellular pool. For example, the lethal action of FU can be expressed even 80 hr after the exposure of cells to FU (31). Therefore even if FU killed only S-phase cells, G_1 and G_2 cells exposed to the drug will accumulate the nucleotide and be killed when the cells enter S.

(*b*) It would be logical to suppose that agents that specifically inhibit DNA synthesis will be lethal to cells in S phase only. ara-C, NSC 107392, and neocarzinostatin are 3 such agents. The first 2 compounds are specifically lethal to cells in S, while neocarzinostatin is more cytotoxic to cells in M and G_1 than in S. The strange behavior of neocarzinostatin might be explained by the observation (27) that cells in G_1, exposed to the drug, were unable to synthesize DNA on reaching S, which probably resulted in cell death. Drug added to cells in S did not inhibit DNA synthesis.

(*c*) Sodium camptothecin inhibits equally both DNA and RNA synthesis and kills cells in all phases of the cycle, although the cells in S were always more sensitive to the drug. In contrast, streptozotocin, Tu, FU, and FUdR at high concentration (30) all inhibited DNA and RNA and/or protein synthesis but were toxic to cells in all phases.

(*d*) Actinomycin D (38) and nogalamycin (8) bind to the DNA template and inhibit RNA synthesis more than DNA synthesis (Table 1). Cells in G_1-S or early S are most sensitive to both these drugs (this paper and Refs. 15 and 33). Fujiwara (17) found that, when actinomycin D was added in G_1, the synthesis of early replicating DNA was inhibited. However, actinomycin D added in the middle of the S phase had little effect on the late replicating DNA. The results indicate that inhibition of RNA synthesis in G_1 led to the inhibition of early replicating DNA, which probably led to cell death.

(*e*) Cells in the S phase were most sensitive to protein synthesis inhibitors, such as pactamycin and sparsomycin. Spalding *et al.* (41) showed that, during replication of chromosomes, S-phase cells also synthesize a new complement of histones. The process of DNA and histone synthesis is tightly coupled so that interruption of histone synthesis results in the inhibition of DNA synthesis. This would account for the sensitivity of cells in S to protein synthesis inhibitors.

(*f*) Our results, together with those of Mauro and Madoc-Jones (33), show that alkylating agents are characterized by toxicity to cells in M, G_1, or G_1-S transition. Whether this common site for the alkylating agents indicates vulnerability of the mitotic chromosomes or of DNA before replication is not known.

In contrast to our results with FUdR, Lozzio (30) reported that FUdR was equally cytotoxic to cells in all phases of the cell cycle except the mitotic stage. However, he did find that 10^{-5} M FUdR killed 97% of the cells in early S, compared to only 70% of the cells killed in G_1 or late S. However, at higher levels of the drug, cells in all phases were equally sensitive.

Our results with BCNU differ from those of Barranco and Humphrey (3), who found that Chinese hamster ovary cells were most sensitive in mid-S phase. Their method differed from ours in 2 aspects:

(*a*) Our DON cells were about 14 times more sensitive than were the ovary cells. Thus, 90% cell kill of asynchronous cells after 1-hr exposure required BCNU, in amounts of 85 and 6 μg/ml for the ovary cells and DON cells, respectively. Therefore, in our phase specificity experiments, DON cells were exposed to BCNU, 2 μg/ml, compared with 100 μg/ml for the ovary cells (3).

(*b*) Their method for synchronizing cells consisted of a double treatment with excess TdR and so differed from ours. Both of us found that cells in the G_1-S border were highly sensitive to BCNU. The cells decreased in sensitivity as they progressed through S, with another zone of great sensitivity in mid-S (3). Here, our results differ from theirs. Whether the difference in the method of synchrony and the levels of BCNU used would account for the difference in our results needs investigation.

Bruce *et al.* (12) differentiated between certain (phase-nonspecific) agents, such as nitrogen mustard and γ-rays, that killed cells in all phases of the cell cycle, and phase-specific agents such as ara-C, that killed cells in a specific phase of the cell cycle. The studies reported here and those of Mauro and Madoc-Jones (33) and others (15, 16) have shown that there is a marked difference in the sensitivity of cells in different phases of the cell cycle toward "phase-nonspecific" (12) agents. Thus, cells at G_1-S transition are 50 times more sensitive to BCNU than the cells in G_2 (Chart 9*b*). The cells in M and G_1 were 250 times more sensitive to ellipticine (Chart 11*b*) than cells in G_2. By proper manipulation, this differential sensitivity may be utilized in cancer chemotherapy.

We suggest that knowlege of the phase specificity of agents may enable us to devise rational combinations of drugs. Thus, 2 drugs inhibiting in the same phase, if given together, will not result in improved cell kill, although they may delay the development of resistance to either drug. However, it is essential that we also know the effect of the drug on progression of the cells through the cell cycle. Thus, it has been suggested that ara-C and HU block cells at the G_1-S interface (2, 19, 25). Our studies (B. K. Bhuyan, W. N. Vorhof,

and T. J. Fraser, paper in preparation) also indicate that NSC 107392 blocks at the G_1-S interface. Thus, it is possible that these drugs (ara-C, HU, and NSC 107392) could be beneficially combined with agents that acted either in M and G_1 (*e.g.*, neocarzinostatin) or at the G_1-S interface (*e.g.*, actinomycin D). Such studies are in progress.

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Cell-Kill Kinetics of Several S-Phase-specific Drugs[1]

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SUMMARY

The drugs [1-β-D-arabinofuranosylcytosine (ara-C), hydroxyurea (HU), 5-hydroxy-2-formylpyridine thiosemicarbazone (5-HP), and camptothecin sodium salt (camptothecin)] considered in this paper markedly inhibit DNA synthesis and are maximally cytotoxic to cells in S phase. In these studies, high-specific-activity thymidine-^{3}H (HSA-TdR-^{3}H) was used as a control compound which killed cells in S but which did not affect the progression of cells into S. The cell-kill kinetics indicated that ara-C, HU, and 5-HP, unlike camptothecin, blocked the L1210 cells from progressing into S in the presence of the drug. We found that L1210 cells that were blocked in G_1 by HU started moving into S immediately after the drug was removed. Therefore, the time interval between two doses of HU that gave maximal cell kill was the same as that for HSA-TdR-^{3}H. However, L1210 cells exposed to ara-C and 5-HP took about 2 hr to recover from the effect of the drug and then progress into S. Therefore, the time interval (between two doses of either ara-C or 5-HP) that gave maximal cell kill was longer than that needed for HSA-TdR-^{3}H. Camptothecin did not block L1210 cells from moving into S and, therefore, the cell-kill kinetics with camptothecin were the same as those with HSA-TdR-^{3}H.

The time for maximal recovery of DNA synthesis by L1210 cells after a single exposure to the drugs was determined. The time for maximal recovery of DNA synthesis correlated well with the interval required for maximal cell kill.

INTRODUCTION

A phase-specific agent will be maximally effective only if it allows cycling cells to enter the cytotoxic phase. Thus, an S-phase-specific agent that blocks the progression of G_1 cells into S will kill only those cells that are in S at the time the drug is added. Sinclair (14, 15) showed that HU[2] has such an effect; namely, the cells in S are killed, while the non-S-phase cells accumulate at the G_1-S boundary. When HU is removed, the accumulated cells proceed synchronously through the cell cycle. ara-C has been shown to have similar effects (7). The protective effect of such self-limiting compounds can be overcome if multiple doses are scheduled so that the cells can recover from drug effects and enter S during the drug-free interval.

The purpose of our studies was to determine the cell-kill kinetics of ara-C, HU, HSA-TdR-^{3}H, 5-HP, and camptothecin with the use of L1210 cells. These drugs were chosen because all of them markedly inhibit DNA synthesis (3), all are maximally cytotoxic to cells in S phase (3), and all (except HSA-TdR-^{3}H) are being studied for efficacy in cancer chemotherapy. L1210 cells were chosen because they are widely used by Drug Research and Development, National Cancer Institute, in screening for antileukemic drugs. Also, the use of L1210 cells enabled us to correlate our *in vitro* data to data obtained *in vivo*. We also determined the optimal time interval between 2 doses of the drug required to give maximal cell kill *in vitro*. The optimal time interval between 2 doses of the drug that gave the maximal increase in life-span of L1210 leukemic mice is reported in a companion paper (13).

MATERIALS AND METHODS

L1210 Cell Methods. L1210 cells were maintained in culture in RPMI Medium 1634 (Grand Island Biological Company, Grand Island, N. Y.) supplemented with fetal calf serum (5%), $NaHCO_3$ (0.075%, w/v), penicillin (0.1 mg/ml), and streptomycin (0.05 mg/ml). The medium was obtained as a freeze-dried powder (without antibiotics, $NaHCO_3$, or serum). The tube dilution assay used to determine cytotoxicity of agents has been described by Buskirk (6). Cell survival after drug treatment was determined by the cloning method of Himmelfarb *et al.* (8). Drug-treated cells were centrifuged at 500 × *g*, and the cells were washed with medium and resuspended at 10^5 cells/ml. The cells were serially diluted in medium, and the final dilution was made in RPMI Medium 1634 containing 20% serum and 0.15% agar. To prepare this soft-agar medium, we autoclaved 1.5% Noble agar (Difco Laboratories, Inc., Detroit, Mich.) in 0.9% NaCl solution. The agar was cooled to 45°, and an aliquot was added to RPMI Medium 1634 containing 20% serum at 45°, to give 0.15% agar. Five ml of the soft agar medium containing suspended cells were dispensed into test tubes and incubated in a humid atmosphere of 8% CO_2 and 92% air at 37°. Each sample was pipetted into 6 tubes for a determination of percentage of cell survival. Colonies were visually counted after 8 to 10 days of incubation. The plating efficiency for L1210 cells was about 50%. In the calculation of percentage survivals, the control (no drug treatment) samples were normalized to 100% survival. The coefficient of variation in determining cell survival was about 15%, the coefficient of variation being the standard deviation expressed as a percentage of the mean.

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[2] The abbreviations and trivial names used are: HU, hydroxyurea (NSC 32065); ara-C, 1-β-D-arabinofuranosylcytosine (cytarabine, NSC 63878); HSA-TdR-^{3}H, high-specific-activity thymidine-^{3}H, 6.7 Ci/mmole; 5-HP, 5-hydroxy-2-formylpyridine thiosemicarbazone (NSC 107392); camptothecin, camptothecin sodium salt (NSC 100880); RPMI, Roswell Park Memorial Institute; TdR-^{3}H, thymidine-^{3}H.

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DON Cell Methods. DON cells, a Chinese hamster fibroblast line (American Type Culture Collection No. CCL16), were grown at 37° in McCoy Medium 5A supplemented with lactalbumin hydrolysate (0.8 g/liter) and fetal calf serum (200 ml/liter). Details of culture maintenance, cell synchronization, and determination of cell survival have been described (3). The plating efficiencies were about 50% for synchronous cells and 70 to 80% for asynchronous cells. In the calculation of survival percentages, the control samples were normalized to 100% survival. The coefficient of variation in determining cell survival was about 15%. Almost all cell-survival experiments were repeated, and the results were found to be reproducible.

Drug Samples. ara-C (Cytosar; cytarabine) was supplied by the Upjohn Company, Kalamazoo, Mich. Camptothecin and 5-HP were obtained from Drug Research and Development, National Cancer Institute, Bethesda, Md. The method for preparing solutions of these drugs has been described (3). HSA-TdR-^{3}H, 6.7 Ci/mmole, was obtained from New England Nuclear, Boston, Mass.

Determination of Macromolecular Synthesis in L1210 Cells. Two-ml samples of L1210 cells (about 5×10^5/ml in RPMI Medium 1634) were preincubated at 37° for 1 hr with various concentrations of the agents studied. Radioactive precursors were then added at the following concentrations: TdR-^{3}H, 2.56 μCi/0.385 μg/ml; uridine-UR-^{3}H, 1.3 μCi/0.13 μg/ml; DL-valine-^{14}C, 0.26 μCi/0.73 μg/ml. After 60 min of incubation, radioactive precursor incorporation was stopped by the addition of excess unlabeled precursor. The amount of precursor incorporation was determined as described previously (3), except that Whatman No. 1 paper discs were used.

DNA Synthesis Recovery Experiments. L1210 cells were subcultured 16 hr prior to use and had reached a cell concentration of approximately 3×10^5/ml at the beginning of the experiment. Fifty-ml portions of cells were added to conditioned culture bottles (*i.e.*, bottles in which L1210 cells had been growing). Agents were added at zero time (Chart 6). Prior to the addition of the agents, a 2.0-ml aliquot of cells was removed and added to 0.1 ml TdR-^{3}H (final concentration, 2 μCi/0.55 μg/ml). After 60 min of incubation, the incorporation of TdR-^{3}H into DNA was determined as described above. This value was taken as the zero-time value equal to 100% of the control DNA synthesis. Sixty min after the addition of agents, DNA synthesis was determined in another 2.0-ml aliquot. At 62 min, 40 ml of the cell suspension were removed and centrifuged (700 × *g* for 2 min at room temperature). Medium that contained drug was removed by aspiration. The cells were washed twice with 15 ml of warm (37°), conditioned medium (*i.e.*, obtained by centrifugation of parallel non-drug-treated cultures) and finally were resuspended in 40 ml conditioned medium, and the incubation was continued. The washing procedure took approximately 20 to 30 min. Cell counts were obtained at various intervals, subsequently. TdR-^{3}H incorporation was determined as described above at various times after the cells were washed free of drug.

RESULTS

Cytotoxicity and Stability of the Drugs. The concentrations needed for 50 and 90% inhibition of L1210 cell growth after 3-day exposure to drug are shown in Table 1. The order of cytotoxicity of the drugs was as follows: camptothecin > ara-C > 5-HP > HU. Only camptothecin was somewhat unstable in medium at 37°.

Inhibition of Macromolecule Synthesis in L1210 Cells. The effect of different doses of drug on the inhibition of DNA synthesis is shown in Chart 1. These data were used to select drug concentrations that gave maximal inhibition of DNA synthesis. The inhibition of DNA, RNA, and protein synthesis at the selected drug concentrations is shown in Table 2. The drugs were used at these concentrations in experiments described below.

L1210 Cell Survival on Short-Term Exposure to Drug. Chart 2 shows the percentage survival of L1210 cells exposed to different levels of drug for 1 hr. In all cases, the slope of the dose-survival curves changed at a certain concentration, indicating that the population consisted of cells with different sensitivities to the drug. With HU, ara-C, and 5-HP, the dose-survival curve decreased to a constant saturation value, indicating that the remaining population was insensitive to the drug (4). With camptothecin, increasing concentrations of the drug killed increasing proportions of the less sensitive population. These agents are maximally cytotoxic to cells in the S phase (3). Therefore, if we correlate the percentage cell survival to the percentage of cells in S (which is about 65% for L1210 cells in culture), we find that (*a*) ara-C, 5-HP, and HU killed cells probably only in the S phase, *i.e.*, about 65% of the cells, and (*b*) high levels of camptothecin killed cells in phases

Table 1
Cytotoxicity and stability of the drugs

Drug	Dose	Growth inhibition[a] (μmoles/ml) 50%	Growth inhibition[a] (μmoles/ml) 90%	Drug left in medium[b] (%) at 2.5 hr	4 hr	7 hr
Camptothecin	0.008	1.8×10^{-5}	4.1×10^{-5}	95	75	60
ara-C	0.007	2.8×10^{-5}	4.8×10^{-5}	98	93	80
5-HP	0.56	2.9×10^{-3}	7.6×10^{-3}		107	100
HU	2.3	3×10^{-2}	8.1×10^{-2}	104	80	82

[a] L1210 cells were incubated with the drugs for three days, following which growth inhibition of drug-treated cells was compared to the controls (6).

[b] Drug was incubated in RPMI Medium 1634 for different periods at 37°. The amount of drug left in the medium was determined by tube-dilution assay (6). Similar results were obtained when L1210 cells in RPMI Medium 1634 were used instead of medium alone.

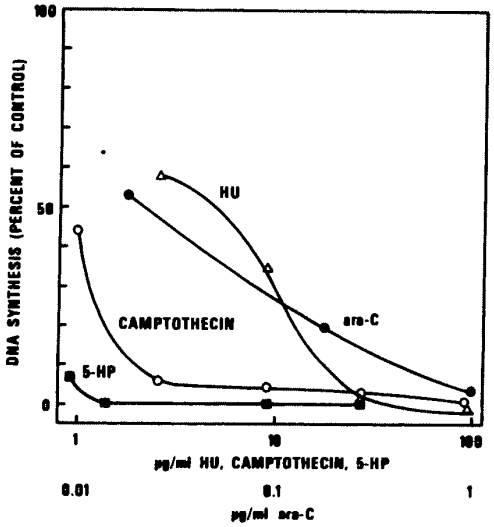

Chart 1. Inhibition of DNA synthesis by L1210 cells with different levels of drugs. The protocol has been described in "Materials and Methods." Cells were incubated with drug and TdR-^{3}H for 1 hr.

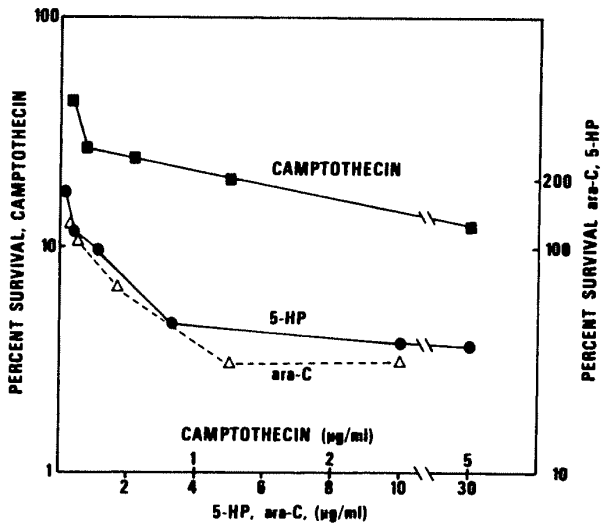

Chart 2. Dose response of L1210 cells to drugs. L1210 cells were exposed to different concentrations of drugs for 1 hr, after which the drug was removed by centrifugation and by washing of the cells. Cell survival was determined as described in "Materials and Methods."

Table 2

Inhibition of macromolecule synthesis by L1210 cells

The protocol has been described under "Materials and Methods." ara-C, HU, and camptothecin were preincubated with cells for 1 hr; radioactive precursors were then added, and the cells were harvested 1 hr later. 5-HP and radioactive precursors were added to the cells together, and the cells were harvested 1 hr later. The control (no drug) cells incorporated TdR-^{3}H, uridine-^{3}H, and valine-^{14}C to give 3100, 7500, and 2200 cpm/10^6 cells, respectively.

Agent	Concentration (μg/ml)	Inhibition of synthesis (%) DNA	RNA	Protein
ara-C	5	97	9	7
HU	30	92	16	11
Camptothecin	10	82	65	16
5-HP	24	96	0	14

other than S, since more than 65% of the cells were killed.

L1210 Cell Survival on Long-Term Exposure to Drug. Chart 3 shows the percentage survival of L1210 cells after exposure to drugs for up to 12 hr. The drug concentrations used were such that an increase in concentration did not result in markedly increased cell kill (Chart 2). The results obtained are compared with the survival of cells exposed to HSA-TdR-^{3}H (6.7 Ci/mmole, 10 μCi/ml). TdR-^{3}H kills S-phase cells subsequent to its incorporation into DNA (5) and presumably does not affect the passage of cells into S at the concentration used. The results indicate the following. (*a*) During the 1st hr, HSA-TdR-^{3}H, HU, ara-C, and 5-HP killed about the same percentage (60 to 70%) of cells (probably the cells in S). In the 1st hr, camptothecin killed more than just S-phase cells. (*b*) Between 1 hr and 4 to 5 hr, the rate of cell kill with ara-C, 5-HP, and HU was much lower than that with HSA-TdR-^{3}H. Thus, at the end of 4 hr, only 2.3% of the cells exposed to HSA-TdR-^{3}H survived, compared with 20 to 30% survival of the cells exposed to HU, ara-C, and 5-HP. After 4 hr, there was

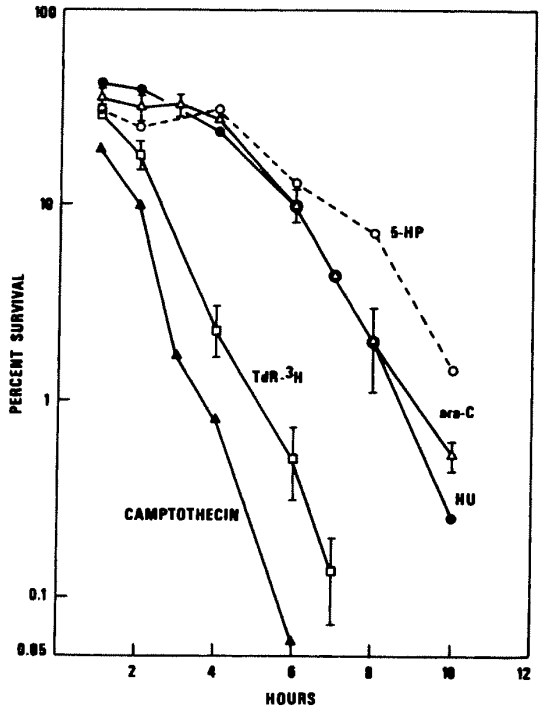

Chart 3. Survival of L1210 cells after exposure to drugs for different periods. After different periods of exposure, aliquots of cells were removed and cell survival was determined. TdR-^{3}H was added to give 20 μCi/ml (6.7 Ci/μmole). ara-C, HU, 5-HP, and camptothecin were present at 5, 300, 30, and 1 μg/ml, respectively. The results shown are the average of 3 to 4 experiments. The mean deviations for ara-C and TdR-^{3}H are shown as examples of mean deviations in these experiments.

a marked increase in the rate of cell kill with cultures exposed to ara-C, 5-HP, and HU. (*c*) The behavior of camptothecin was different from that of the 3 drugs mentioned above. The rate of cell kill with camptothecin paralleled that seen with HSA-TdR-^{3}H.

These experiments suggested that, unlike camptothecin, HU, ara-C, and 5-HP delay the entry of non-S-phase cells into the drug-sensitive S phase. A similar plateau in the survival curve of mammalian cells exposed to HU and ara-C was previously reported (1, 7, 10) and was explained as indicating a reduced rate of flow of G_1 cells into the drug-sensitive S phase. In order to determine directly the effect of HU, 5-HP, ara-C, and camptothecin on the rate of entry of cells into S, we performed the following experiments. Since a synchronized population of L1210 cells was not available, synchronized DON cells were used.

Studies with Synchronized DON Cells. These experiments were based on the following facts: (*a*) Mitotic DON cells stay in G_1 for about 2 hr, and (*b*) all of the drugs studied are most cytotoxic to cells in the S phase (3).

In the 1st series, the drug was added to the mitotic cells immediately after the cells were planted, and the drug was left in contact with the cells for the total period of the experiment. If the drug inhibited the entry of cells into S, then the cells would not be killed even after exposure to the drug for 4 to 5 hr, *i.e.*, by the time that the cells would normally be in S.

In the 2nd series, the cells were exposed to the drug for 1 hr at different times after they were planted. When the drug was added to cells in G_1, a small proportion of the cells were killed, partly due to contamination of G_1 cells with S cells. However, if the drug was added to cells after they entered S, then marked cell kill was seen with these S-phase-specific agents.

Therefore, a difference in cell survival between mitotic cells exposed to the drug continuously (1st series) and S-phase cells exposed to the drug (2nd series) would indicate that the drug affects the rate of entry of cells into S phase.

The results shown in Chart 4 indicate that ara-C, 5-HP, and HU (in contrast to HSA-TdR-^{3}H and camptothecin) inhibit the entry of cells into S. HSA-TdR-^{3}H was used as a control compound which kills cells only in the S phase and which does not affect the passage of cells into S. HSA-TdR-^{3}H killed about 18% of the cells during the 1st hr, indicating partial contamination of the mitotic cells with S-phase cells. By 2 hr, cells had started entering S and, by 4 hr, about 90% of the cells were in S and were killed. The percentage cell survival was the same when cells were continuously exposed to HSA-TdR-^{3}H from zero time as when HSA-TdR-^{3}H was added for 1-hr periods at different times after planting. Such a result would be expected for an agent that did not affect the entry of G_1 cells into S. Camptothecin behaved in a manner similar to that of HSA-TdR-^{3}H, indicating that this drug did not affect the rate of entry of G_1 cells into S.

With 5-HP, there was little cell kill during the 1st 2 hr after mitotic cells were planted (*i.e.*, of the cells in G_1). When the drug was added for 1 hr to S-phase cells (*i.e.*, 4 hr after planting), 88% of the cells were killed. However, when the drug was added to cells at zero hr and were left in contact with the cells for 5 hr, only 30% of the cells were killed. This suggests that 5-HP inhibited the entry of cells into S. Similar results were obtained with HU and ara-C, and similar results were obtained if, instead of adding the drugs to mitotic cells, we added the drugs shortly before the cells were due to enter S. This indicated that the drugs were not acting by blocking mitosis or lengthening the G_1 phase.

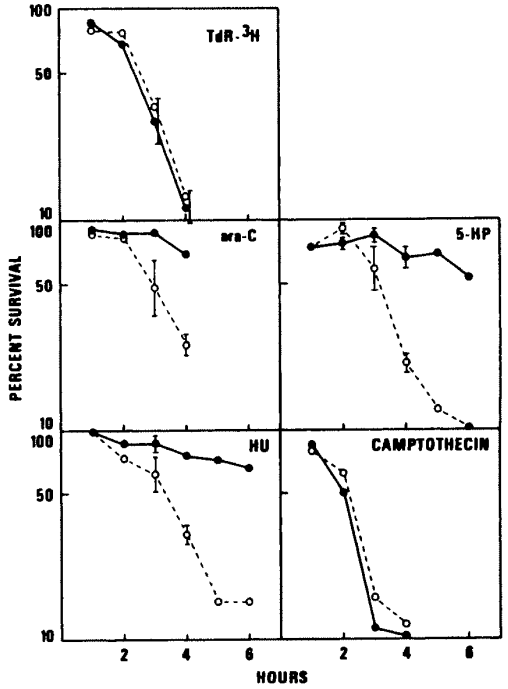

Chart 4. Comparison of cell survival when drug is added to mitotic DON cells immediately or at different times after planting. Mitotic DON cells were planted in 3-oz prescription bottles in 10 ml of medium. After drug exposure, the cell monolayer was washed and the cells were detached with trypsin (1 mg/ml). The cells were diluted in medium and planted in Linbro plates, and colonies were counted after 7 to 8 days of incubation. Results shown are the average of 2 to 3 experiments (mean ± S.D.); ——, drug was added to mitotic cells immediately after planting and was left in contact for the period of the experiment. Cells were harvested at different times, and cell survival was determined. - - - -, mitotic cells were planted, and the cells were exposed to drug for 1 hr at different times after planting. After drug exposure, cells were harvested and cell survival was determined. ———— (*4-hr time point*), mitotic cells were exposed to drug from 0 to 4 hr; - - - - (*4-hr time point*), drug was added 3 hr after mitotic cells were planted, and the cells were exposed to drug for 1 hr.

Effect on L1210 Cells of Intermittent Exposure to Drug. Previous experiments showed that, in the presence of ara-C, HU, and 5-HP, the progression of G_1 cells (both DON and L1210) into S was inhibited. Thus, the non-S-phase cells were protected from the cytotoxic effects of the drug. This protective effect can be overcome by giving the drug in multiple doses, with the intervals designed to give enough time for the non-S-phase cells to recover and enter S during the drug-free period. The experiments reported here (Charts 5 and 6) show the cell kill obtained after 2 exposures (each 1 hr in duration), with the exposures separated by different time intervals.

When the agents were given in 2 divided doses, the time

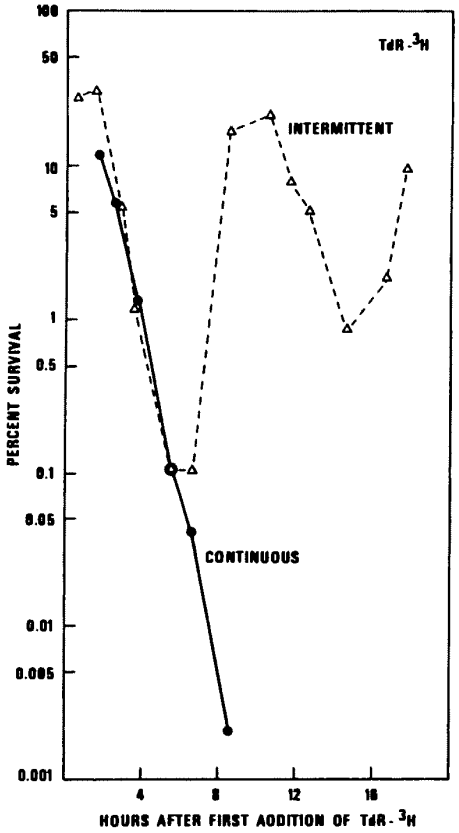

Chart 5. Survival of L1210 cells when the cells were exposed to HSA-TdR-^{3}H continuously (——) or to 2 divided doses added at different intervals (- - - -). ——, HSA-TdR-^{3}H (10 μCi/ml, 6.7 Ci/mmole) was added to L1210 cells in suspension. Aliquots were taken at different times, and thymidine (10 μg/ml) was added to dilute the HSA-TdR-^{3}H. Cells were centrifuged, washed, diluted in medium, and planted to determine cell survival. - - - -, cells were exposed to HSA-TdR-^{3}H (10 μCi/ml, 6.7 Ci/mmole) for 1 hr, following which thymidine (10 μg/ml) was added. The cells were centrifuged, washed, and replanted at approximately the same cell concentration in fresh medium. The cells were exposed to HSA-TdR-^{3}H (10 μCi/ml, 6.7 Ci/mmole) for a 2nd 1-hr period at various times after the 1st dose. Then the cells were washed and cell survival was determined. *Time scale*, time between the beginning of the initial drug exposure to the middle of the 2nd drug exposure.

intervals for maximal cell kill were HSA-TdR-^{3}H, 5.5 and 14.5 hr; HU, 5.5 hr; camptothecin, 5.5 and 15.5 hr; ara-C, 7.5 and 18.5 hr; and 5-HP, 7.5 and 17.5 hr. The time intervals for minimal cell kill were: HSA-TdR-^{3}H, 10.5 hr; HU, 10.5 hr; camptothecin, 9.5 hr; ara-C, 12.5 hr; and 5-HP, 13.5 hr. With all of the drugs (except camptothecin), 2 exposures of 1-hr duration, separated by the optimal time interval, killed more cells than did continuous exposure to the drug for 6 hr. Thus, 6 hr of continuous exposure to ara-C, HU, or 5-HP killed about 90% of the cells, compared to greater than 99% cell kill achieved with 2 doses, given at the optimal interval (compare Charts 3 and 6).

Chart 6 also shows the time taken for L1210 cells to recover their capacity to synthesize DNA after a single dose of each drug. Immediately after 1 dose of each drug, DNA synthesis is almost completely inhibited. After removal of the drug, TdR-^{3}H is incorporated into (*a*) the cells in S at the time of exposure to drug and (*b*) the cells entering S from G_1. Therefore, the time for maximal recovery of DNA synthesis indicates (in a very approximate manner) the time taken for non-S-phase cells to enter S after removal of the drug. This should be the optimal time for a 2nd dose of an S-phase-specific drug to obtain maximal cell kill.

The interval between two 1-hr exposures to the agent, for optimal recovery of DNA synthetic capacity, corresponded well with the interval for maximal cell kill. With camptothecin and 5-HP, DNA synthetic capacity reached levels greater than those observed with control samples.

DISCUSSION

Previous studies with synchronous DON cells showed that only ara-C, HU, and 5-HP were specifically cytotoxic to cells in S, while camptothecin also killed cells in G_1 and G_2 at high concentrations (3, 12). The dose-survival curves (Chart 1) with L1210 cells indicated that ara-C, HU, and 5-HP were specifically cytotoxic to cells in a certain phase (probably S)

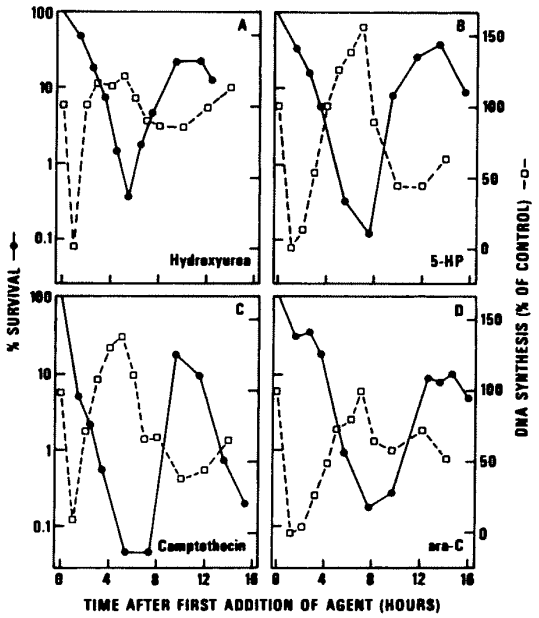

Chart 6. Survival of L1210 cells exposed to 2 divided doses at different intervals (——). The protocol for this study is outlined in Chart 5 (except that different drugs replaced HSA-TdR-^{3}H, and thymidine was not added). The agents were used at the following levels: HU, 300 μg/ml; 5-HP, 30 μg/ml; camptothecin, 1 μg/ml; and ara-C, 5 μg/ml. Recovery of the ability to synthesize DNA after exposure to 2 divided doses of the drug (- - - -). The detailed protocol is described in "Materials and Methods." In these experiments, the control cells incorporated TdR-^{3}H to give approximately 6 × 10^4 cpm/10^6 cells. The agents were used at the following levels: HU, 30 μg/ml; 5-HP, 24 μg/ml; camptothecin, 10 μg/ml; and ara-C, 5 μg/ml. *Time scale*, time between the beginning of the initial drug exposure to the time of addition of TdR-^{3}H.

of the cell cycle, while camptothecin killed cells in other phases.

We compared the time course of cell kill with ara-C, HU, and 5-HP to that of HSA-TdR-^{3}H (Chart 2). HSA-TdR-^{3}H was chosen as a reference S-phase-specific agent that does not block the progression of non-S-phase cells into S. The following discussion justifies this choice.

The cell-cycle times and percentage of cells in each phase for L1210 cells in culture were found to be as follows: G_1, 1.6 hr and 17% of total cells; S, 8.2 hr and 69%; G_2, 1.9 hr and 11%; and M, 0.5 hr and 3%. If we assume that all S-phase cells are killed by a short exposure to HSA-TdR-^{3}H and that HSA-TdR-^{3}H does not affect the progression of G_1 cells into S, then we can calculate the cell kill that can be expected after increasing periods of exposure to TdR-^{3}H. The expected percentage of cell kill would be approximately equal to $100 \times [(T_S + \text{length of exposure time})/T_C]$, where T_S is the S time period and T_C is the cell-cycle time. If we also take into account the increase in cell number that occurs as the viable G_2 and M cells divide, then the percentage cell kill after 1, 2, 3, and 4 hr of exposure to TdR-^{3}H would be 75, 80.5, 88.2, and 98.2, respectively. These calculated values correspond closely with the observed values (Chart 3). This justifies our use of HSA-TdR-^{3}H as an S-phase-specific agent that does not block the progression of G_1 cells into S.

We found that the continuous presence of ara-C, HU, and 5-HP delayed the entry of non-S-phase cells into the drug-sensitive S phase (Charts 3 and 4). Other workers using L-cells also observed a rapid initial cell kill by HU (1) and by ara-C (7), followed by a plateau phase with a slow rate of cell kill. Similar results were also obtained by Kim *et al.* (10) when they exposed HeLa cells to HU. The plateau in the survival curves of cells exposed to HU and ara-C resulted from a reduced rate of flow of G_1 cells into the drug-sensitive S phase (1, 7, 10). Sinclair (14, 15) also suggested that cells not in S phase at the time of HU addition are prevented from entering S, due to a lack of DNA synthesis, and are thereby protected from the lethal effects of the drug. Bertalanffy and Lindsay-Gibson (2) showed that 4 daily injections of 12.5 mg of ara-C per kg produced partial synchronization of B16 melanoma and Ehrlich ascites tumor cells *in vivo*. Such synchrony would be expected if the cells were blocked at the end of G_1 in the presence of the drug and were released in unison when the drug was metabolized and removed from the body fluids. Also, Tobey and Crissman (19) showed that G_1-arrested cells released from the isoleucine-mediated arrest start entering S 4 hr after isoleucine addition. However, similar cells treated for 10 hr with HU (10^{-3} M) or ara-C (5 μg/ml) contained the amount of DNA expected of G_1 cells. These and other studies (18) led these authors to conclude that, although neither ara-C nor HU *completely* prevented cells from initiating DNA synthesis, they grossly decreased the progression of cells from G_1 to S.

Skipper *et al.* (16) found that ara-C (15 to 20 mg/kg), given at 3-hr intervals on Days 2, 6, 10, and 14, cured a substantial number of leukemic mice. This is apparently contradictory to the protective effect (by blocking non-S-phase cells from entering S) of ara-C discussed above. However, this can be explained by the decrease in ara-C level in the plasma, with a half-life of about 15 min (16), to about 1 μg/ml 1 hr after injection. Karon and Shirakawa (9) have shown that the transit rate of cells from G_1 to S or from G_2 to G_1 was not affected when DON cells were exposed to 1 μg ara-C per ml for 1 hr. Therefore, it is possible that L1210 cells exposed to about 1 μg ara-C per ml for 1 hr would be able to progress into S and would be killed.

In contrast to the self-limiting effect of ara-C, HU, and 5-HP, camptothecin did not affect the progression of G_1 cells into S. Tobey (17) showed that initiation of genome replication could occur in the presence of this drug.

Maximal cell kill was obtained when 2 doses of TdR-^{3}H were given 5 or 14 hr apart, and minimal cell kill resulted when the doses were 10 hr apart. If we assume that the L1210 cell population is homogeneous with respect to cell-cycle times and the lengths of various phases, the expected interval for maximal and minimal cell kill can be calculated. Since the S cells are killed by the 1st dose, we need only consider the position of the non-S- ($G_1 + M + G_2$) phase cells at different times after the 1st dose. Since $G_1 + M + G_2$ is 4 hr (see the cell-cycle times given previously), all of the $G_1 + M + G_2$ cells will be in S between 4 and 8.2 hr after the 1st dose and will be killed by the 2nd dose. Between 8.2 and 12.2 hr after the 1st dose, the $G_1 + M + G_2$ cells will be leaving S and will be insensitive to the 2nd dose. Between 12.2 and 16.2 hr, all of the $G_1 + M + G_2$ cells will be entering S and will be sensitive to the 2nd dose. These calculated values compare quite well with the observed values for HSA-TdR-^{3}H, if one considers that the cells are not completely homogeneous with respect to cell-cycle times and with the time taken to wash off HSA-TdR-^{3}H. Intervals for maximal and minimal cell kill with HU and camptothecin were similar to those with TdR-^{3}H. Thus, although cells are blocked from progressing into S in the presence of HU, once the HU is removed, $G_1 + M + G_2$ cells immediately start entering S. However, with ara-C and 5-HP, $G_1 + M + G_2$ cells are slowed down in their entry into S, even after the drug is removed. Therefore, our results indicate that, after removal of the 1st dose of ara-C and 5-HP, it takes 2 hr longer for $G_1 + M + G_2$ cells to enter S than after removal of HU, camptothecin, or HSA-TdR-^{3}H. Karon and Shirakawa (9) found that after exposure of DON cells to 10 μg ara-C per ml for 1 hr, the subsequent transit rate of G_1 cells into S, and of S cells to G_2 was decreased, although the progression of G_2 cells to mitosis was not affected. A block in the progression of S cells to G_2 would not affect the cell kill with the 2nd dose, since the cells in S would already be killed by the 1st dose.

These results with cell-kill experiments were supported by the measurements of the time course of recovery of DNA synthesis in cells exposed to the drugs. These measurements indicate (although in a very approximate manner) the time taken for non-S-phase cells to enter S after the drug is removed. The amount of TdR-^{3}H incorporated following the removal of the drug consists of (*a*) incorporation by the cells (in S) "injured" by the S-phase-specific drugs and (*b*) the incorporation by cells entering S. Regarding the 1st point, Sinclair (15) showed that S-phase cells, destined to die after exposure to HU, resynthesized DNA after the drug was removed. The rate of recovery of DNA synthesis by the injured cells may vary depending on their position in S and the period of recovery. Also, the rate of DNA synthesis in the cells entering S will vary depending on the position of these cells in

S at different times after drug is removed. However, in spite of these factors, there is a reasonable correlation between the optimal dose interval and the interval for maximal recovery of DNA synthesis.

We have shown that 2 doses of an S-phase-specific drug given at proper intervals are more cytotoxic toward L1210 cells in culture than the same dose maintained continuously for approximately 8 hr (compare Charts 3 and 6). This is particularly true if the cells are blocked from entering the sensitive phase in the presence of the drug. However, it is important to note that, during continuous exposure to these latter agents, the rate of cell kill increases rapidly after the 1st 4 to 6 hr of exposure. Indeed, it is possible to achieve greater cell kill with long exposures (*i.e.*, more than 10 to 12 hr) than it is with intermittent exposures. These results taken together would suggest that in the clinical treatment of leukemia with ara-C, for example, properly spaced intermittent treatment would be superior to short-term infusions but would be inferior to long-term infusions. The clinical data bearing on this point is quite ambiguous (11). No one regimen was shown to be clearly superior. It is, of course, impossible to directly extrapolate "therapeutic regimens" in cell culture experiments to the clinical situation. The effectiveness and, in particular, the therapeutic index of any dosage regimen *in vivo* will depend on a number of additional factors, not the least of which are the kinetic differences between the tumor cells and the normal cells of the host. In spite of this, as shown in the companion paper (13), the validity of many of the results of these studies *in vitro* has at least been confirmed in a murine experimental tumor system.

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ORIGINAL ARTICLE

DNA Methylation Markers and Early Recurrence in Stage I Lung Cancer

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ABSTRACT

BACKGROUND

Despite optimal and early surgical treatment of non–small-cell lung cancer (NSCLC), many patients die of recurrent NSCLC. We investigated the association between gene methylation and recurrence of the tumor.

METHODS

Fifty-one patients with stage I NSCLC who underwent curative resection but who had a recurrence within 40 months after resection (case patients) were matched on the basis of age, NSCLC stage, sex, and date of surgery to 116 patients with stage I NSCLC who underwent curative resection but who did not have a recurrence within 40 months after resection (controls). We investigated whether the methylation of seven genes in tumor and lymph nodes was associated with tumor recurrence.

RESULTS

In a multivariate model, promoter methylation of the cyclin-dependent kinase inhibitor 2A gene *p16*, the H-cadherin gene *CDH13*, the *Ras* association domain family 1 gene *RASSF1A*, and the adenomatous polyposis coli gene *APC* in tumors and in histologically tumor-negative lymph nodes was associated with tumor recurrence, independently of NSCLC stage, age, sex, race, smoking history, and histologic characteristics of the tumor. Methylation of the promoter regions of *p16* and *CDH13* in both tumor and mediastinal lymph nodes was associated with an odds ratio of recurrent cancer of 15.50 in the original cohort and an odds ratio of 25.25 when the original cohort was combined with an independent validation cohort of 20 patients with stage I NSCLC.

CONCLUSIONS

Methylation of the promoter region of the four genes in patients with stage I NSCLC treated with curative intent by means of surgery is associated with early recurrence.

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SURGERY WITH CURATIVE INTENT IS THE standard of care for patients with stage I non–small-cell lung cancer (NSCLC), yet notwithstanding advances in treatment, the dissemination of tumor cells outside the area of curative resection is a leading cause of relapse.[1-3] Despite surgery, approximately 30 to 40% of patients with NSCLC who have discrete lesions and histologically negative lymph nodes (stage I cancer; T1–2N0, according to the tumor–node–metastasis [TNM] classification criteria) die of recurrent disease.[4-6] Many of these recurrences are systemic, making it likely that such patients had occult micrometastases beyond the margins of surgical resection.[4]

Epigenetic gene silencing is a molecular mechanism of silencing a gene by methylating its promoter region. Epigenetic silencing is involved in the initiation and progression of several types of cancer, including lung cancer.[7,8] The detection of epigenetic alterations with the use of a method like the methylation-specific polymerase-chain-reaction (PCR) assay[9] may allow for the molecular staging of cancer. With the methylation-specific PCR assay, relatively few genes are required to analyze each type of cancer.[9] The method can detect occult micrometastases in lymph nodes from patients with esophageal,[10] colorectal,[11] gastric,[10] prostate,[12] or lung cancer,[10,13] but its value for predicting the recurrence of early-stage, resected NSCLC has not been examined.

We designed a nested case–control study of early-stage NSCLC (T1–2N0) to test the association between clinical outcome and the DNA methylation status of tumor, regional lymph nodes confined to the pleural space, and mediastinal lymph nodes. We studied seven genes: the cyclin-dependent kinase inhibitor 2A gene *p16*, the H-cadherin gene *CDH13*, the adenomatous polyposis coli gene *APC*, the *Ras* association domain family 1 gene *RASSF1A*, the O^6-methylguanine–DNA methyltransferase gene *MGMT*, the PYD and CARD domain–containing gene *ASC*, and the death-associated protein kinase 1 gene *DAPK*. These genes are thought to be important in the biologic development of lung cancer and are frequently methylated in lung cancer.[14-18] We hypothesized that the methylation-specific PCR assay could be used to define patterns of DNA methylation that can delineate the behavior of the primary tumor and to detect micrometastases in histologically negative lymph nodes. Our results show that aberrant patterns of promoter methylation in the primary tumor, and in regional and mediastinal lymph nodes, can be used to identify patients with stage I NSCLC who have an increased risk of recurrence.

METHODS

PATIENTS

Evidence of recurrent disease was evaluated in 715 patients with pathologically verified stage I (T1–2N0) cancer who received a diagnosis of NSCLC (codes 162.3 to 162.9 of the *International Classification of Diseases, Ninth Revision, Clinical Modification*) and who underwent lobectomy or greater resections at the Johns Hopkins Hospital between January 1, 1986, and July 31, 2002. The case patients were 71 patients at our institution in whom the tumor recurred within 40 months after surgery, by which time approximately 80% of NSCLC recurrences occur.[4] Follow-up of all 71 case patients was performed at Johns Hopkins Hospital with the use of radiographic imaging and, usually, histologic verification of recurrence. On the basis of age, NSCLC stage, date of surgery (±5 years), and sex, we matched the case patients to 158 controls with stage I NSCLC in whom there was no recurrence during the 40-month follow-up period. In this phase of the study, tissue samples from 51 of the 71 case patients and 116 of the 158 matched controls were available for methylation analysis. Seven of the 116 controls had a recurrence more than 40 months after surgery. Neither case patients nor controls received adjuvant chemotherapy; between 1986 and 2002, guidelines did not recommend adjuvant therapy for patients with stage IB NSCLC.[19,20]

All cases of cancer were staged according to the revised TNM classification criteria,[5] including the histologic status of mediastinal lymph nodes sampled from levels II, IV, VII, VIII, IX, and X on the right side and levels V, VI, VII, VIII, and IX on the left side. Regional lymph nodes confined to the pleural space were resected en bloc with the tumor. Samples from case patients had no macroscopically or microscopically positive surgical margins, and the patients had had a lobectomy or greater resection.[21] We also evaluated 162 paraffin-embedded tissue blocks from the 20 patients in the validation cohort (11 case patients and 9 matched controls). Of these 20 patients, 18 had undergone resection at our institution after

August 2002. This study was approved by the Johns Hopkins Institutional Review Board. The requirement of written informed consent was waived.

PREPARATION OF TUMOR AND LYMPH-NODE SPECIMENS

All specimen blocks were procured from pathology archives en masse in a blinded fashion with regard to whether they were obtained from case patients or controls. There were no differences in the distribution of tumor and lymph nodes or number of samples per patient between the case patients and controls. Specimens were labeled with study-specific coded identifiers only; laboratory investigators had no knowledge of the patient's group or the source tissue of the DNA. DNA was extracted from a pool of three sequential sections, each 10 μm in thickness, from unstained, paraffin-embedded slides of resected tumors, regional lymph nodes, or mediastinal lymph nodes. For each sample of tumor or lymph-node tissue, adjacent sections were stained with hematoxylin and eosin for histologic confirmation of the presence or absence of cancer. Unstained tissue sections were deparaffinized, and DNA was extracted as described previously.[22] The concentration of DNA was measured spectrophotometrically, and 1 μg of DNA was denatured with the use of sodium hydroxide and modified with the use of sodium bisulfite. We then purified the DNA samples by using Wizard DNA purification resin (Promega), treated them again with sodium hydroxide, precipitated them with ethanol, and resuspended them in water.

METHYLATION-SPECIFIC PCR ASSAY

DNA methylation was evaluated with the use of the methylation-specific PCR assay, performed by three persons working independently. Each of them extracted DNA and performed all steps of the assay separately.[23] A combined total of 889 samples of tumor and lymph-node tissues were examined. A multiplex-nested methylation-specific PCR assay was used for all samples, as described previously.[24] The nested method initially amplifies bisulfite-modified DNA with the use of flanking PCR primers, without preferentially amplifying methylated or unmethylated DNA. The resulting fragment is then used as the template for the methylation-specific PCR assay. Primer sequences and PCR conditions for *p16*, *MGMT*, *DAPK*, *RASSF1A*, *CDH13*, *ASC*, and *APC* have been described previously,[15,17,24-26] including conditions optimized to achieve specific detection of methylation in tumor tissue but not in normal lymphocytes (Table 1 in the Supplementary Appendix, available with the full text of this article at www.nejm.org).[15,17,24-26] Placental DNA treated with *Sss*I methyltransferase (New England Biolabs) was used as a positive standard. DNA from normal lymphocytes and water (bisulfite-modified water and unmodified water) were used as negative standards. PCR products were separated on 2% agarose gel or 6% nondenaturing polyacrylamide gel and were visually scored as methylated or unmethylated according to the presence or absence of a PCR product, respectively (Fig. 1 in the Supplementary Appendix).[23,25,27] If any tumor block or lymph-node specimen was positive for methylation, all of the primary tumor or all associated lymph nodes in that nodal basin, respectively, were scored as positive.

STATISTICAL ANALYSIS

We verified histologic results and deaths or recurrent disease during the follow-up period by reexamining the original hospital records. The primary end point was time to recurrent local or distant disease, measured from the date of surgery to the time of cancer-related death or censoring. Data for controls who were alive and had no evidence of disease at the end of the study were censored for recurrence or death. All deaths of case patients were cancer-related, and no controls were lost to follow-up. Associations among prognostic factors, presence or absence of recurrence, and patient group were assessed by means of univariate and multivariate logistic-regression analysis. The association of risk factors with time-to-event or time-to-censoring end points was analyzed with the use of the log-rank test. Results of all models are reported as odds ratios with 95% confidence intervals. All statistical calculations were performed with the use of Stata statistical software. Two-sided P values of less than 0.05 were considered to indicate statistical significance.

We hypothesized that 40% or more of the case patients would have microscopic disease in the resected lymph nodes, as compared with 20% or less of controls, yielding an odds ratio of 2 for case patients. Under these assumptions, the study would have a statistical power of 80% to detect a

Table 1. Baseline Characteristics of the 187 Patients.*

Characteristic	Original Cohort (N=167)		Validation Cohort (N=20)
	Case Patients (N=51)	Controls (N=116)	
Age — yr			
Median	64	67	66
Interquartile range	58–71	60–72	57–72
Sex — no. (%)			
Male	24 (47.1)	54 (46.6)	8 (40.0)
Female	27 (52.9)	62 (53.4)	12 (60.0)
Race — no. (%)†			
White	43 (84.3)	96 (82.8)	15 (75.0)
Black	6 (11.8)	19 (16.4)	5 (25.0)
Other	2 (3.9)	1 (0.9)	0
Stage — no. (%)			
IA (T1N0)	26 (51.0)	75 (64.7)	9 (45.0)
IB (T2N0)	25 (49.0)	41 (35.3)	11 (55.0)
Tumor diameter — no. (%)			
≤3 cm	25 (49.0)	72 (62.1)	13 (65.0)
>3 cm	26 (51.0)	44 (37.9)	7 (35.0)
Surgical procedure — no. (%)			
Lobectomy	46 (90.2)	95 (81.9)	20 (100)
Pneumonectomy or bilobectomy	4 (7.8)	4 (3.4)	0
Sublobar resection	1 (2.0)	17 (14.7)	0
Histologic characteristics — no. (%)‡			
Adenocarcinoma	30 (58.8)	62 (53.4)	15 (75.0)
Squamous-cell	15 (29.4)	42 (36.2)	4 (20.0)
Other	6 (11.8)	12 (10.3)	1 (5.0)
Median ASA physical-status score§	3	3	3
Smoking status — no. (%)			
Current or former smoker	43 (84.3)	102 (87.9)	20 (100)
Nonsmoker	8 (15.7)	12 (10.3)	0
Unknown	0	2 (1.7)	0

* The case patients were matched with the controls on the basis of age, sex, NSCLC stage, and date of surgery (±5 years). Because of rounding, percentages may not total 100.
† Race was self-reported.
‡ "Adenocarcinoma" includes bronchioloalveolar carcinoma and adenosquamous histologic features. "Other" includes large-cell, basaloid, and mucoepidermoid histologic features.
§ Physical status was graded according to the American Society of Anesthesiologists (ASA) Physical Status Classification System; scores range from 1 to 6, with higher scores indicating more severe disease. A score of 3 corresponds to a patient with severe systemic disease.

significant effect among 168 case patients and controls, matched in a two-to-one ratio.

The authors designed the study; gathered, interpreted, and held the data; wrote the paper; made the decision to publish; and vouch for the completeness and accuracy of the data. There were no agreements concerning confidentiality of data between OncoMethylome Sciences and the authors.

Table 2. Prevalence of Gene Methylation in Tumor or Regional or Mediastinal Lymph Nodes in the Original Cohort, According to Gene.*

Methylated Gene	Tumor		P Value	Regional Lymph Nodes		P Value	Mediastinal Lymph Nodes		P Value
	Controls (N=104)	Case Patients (N=50)		Controls (N=82)	Case Patients (N=41)		Controls (N=56)	Case Patients (N=34)	
	percent			*percent*			*percent*		
MGMT	36.1	34.7	0.87	29.5	37.5	0.38	35.8	44.1	0.44
ASC	34.9	38.8	0.65	27.2	29.3	0.81	42.9	44.1	0.91
DAPK	35.3	36.0	0.93	41.5	42.5	0.91	30.8	39.4	0.41
APC	34.0	36.0	0.81	18.7	13.5	0.48	13.0	29.4	0.06
RASSF1A	35.9	50.0	0.10	16.5	12.8	0.61	9.6	20.6	0.15
p16	26.0	52.0	0.001	13.7	35.0	0.009	16.7	48.5	0.001
CDH13	22.8	38.8	0.04	19.5	32.5	0.12	25.0	46.9	0.04

* P values were calculated with the use of the chi-square test for homogeneity.

RESULTS

CHARACTERISTICS OF THE PATIENTS

Clinical and demographic variables were similar in case patients and controls (Table 1). On the basis of the American Society of Anesthesiologists Physical Status Classification System, case patients and controls were found to be equally fit for surgery. The most frequent site of recurrence was the ipsilateral lung (in 45.1% of patients), followed by metastasis to bone (13.7%), brain (11.7%), and mediastinum (11.7%). Although 14.7% of controls underwent sublobar resections, all pulmonary resections in these patients were done with curative intent.

RISK OF RECURRENCE ACCORDING TO CLINICAL PREDICTORS

The covariates of pathological stage, age, sex, histologic characteristics of the tumor, smoking status, and race were not associated with the risk of recurrence in patients with histologically negative lymph nodes (Table 2 in the Supplementary Appendix). Although pathological tumor stage showed the strongest association with recurrence, independently of other covariates, the association was not significant. For example, patients with stage IB disease (according to the 1986 classification of the American Joint Committee on Cancer[28]) had an adjusted odds ratio for recurrence of 1.71 (95% confidence interval [CI], 0.86 to 3.41; P=0.13), as compared with patients with smaller tumors and no pleural invasion (stage IA disease) (Table 2 in the Supplementary Appendix).

GENE METHYLATION AND RECURRENCE

Methylation profiles of the seven genes were obtained from 727 of the 731 paraffin blocks, from 167 cases and controls. As compared with controls, the largest differences in the univariate distribution among case patients of the frequency of methylation in any type of tissue were found in four genes — *p16*, *CDH13*, *RASSF1A*, and *APC* — especially in tumors or mediastinal lymph nodes (Table 2). When *p16* or *CDH13* was methylated in the primary tumor, the adjusted odds ratio for recurrence was 3.50 (95% CI, 1.65 to 7.41; P=0.001) and 2.12 (95% CI, 0.98 to 4.59; P=0.06), respectively (Fig. 1 and Table 3). When these same genes were methylated in regional lymph nodes, the odds ratio was 3.62 (95% CI, 1.41 to 9.32; P=0.008) and 1.99 (95% CI, 0.81 to 4.88; P=0.13), respectively. If methylation of *p16* or *CDH13* was found in mediastinal lymph nodes, the odds of recurrence was 4.67 (95% CI, 1.53 to 14.42; P=0.007) and 3.98 (95% CI, 1.22 to 13.01; P=0.02), respectively (Fig. 1 and Table 3). Methylation of *RASSF1A* or *APC* in the tumor or mediastinal nodes was not significantly associated with recurrence (Fig. 1).

The six possible pairs of these four genes were examined for an association with recurrence. Among these pairs, four had a significant association with recurrence in at least one type of tissue: *p16* and *CDH13*, *CDH13* and *APC*, *APC* and *p16*, and *RASSF1A* and *p16* (Table 3 and Fig. 1). Methylation of the gene pair *p16* and *CDH13* in the primary tumor alone was associated with an odds ratio for recurrence of 8.00 (95% CI, 2.50 to 25.51; P<0.001) for the case patients as

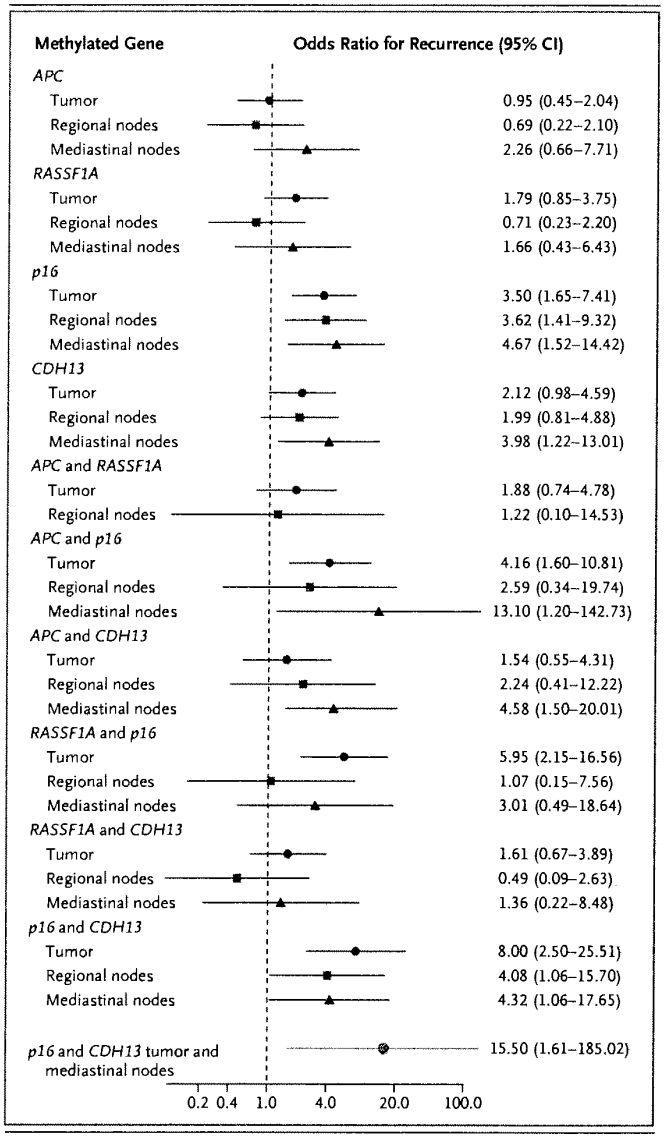

Figure 1. **Odds Ratios for Recurrence among Case Patients as Compared with Controls, According to Methylated Gene and Site.**

Multivariate logistic-regression analysis was performed with the use of data for the four genes that had the largest univariate differences in distribution with regard to methylation: the adenomatous polyposis coli gene *APC*, the *Ras* association domain family 1 gene *RASSF1A*, the cyclin-dependent kinase inhibitor 2A gene *p16*, and the H-cadherin gene *CDH13*. The prognostic value of each gene was adjusted for stage (IA or IB), age, sex, race (white or black), histologic feature of the tumor (adenocarcinoma, squamous-cell, or other), and smoking status (current or former smoker or nonsmoker) and then graphed as a forest plot. Among single genes, the methylation of either *p16* or *CDH13* was associated with the most significant odds ratios of recurrence, regardless of the type of tissue. Methylation of either *RASSF1A* or *APC* in the primary tumor or in mediastinal lymph nodes was associated with an elevation in the odds of recurrence that was not significant. Patients with methylation of both *p16* and *CDH13* in both tumor and mediastinal lymph nodes had a significantly higher odds of lung-cancer recurrence than those without methylation of this pair of genes (P=0.03).

compared with the controls; when methylation of the two genes was found in both the tumor and the mediastinal lymph nodes, the estimated odds ratio for recurrence was 15.50 (95% CI, 1.61 to 185.02; P=0.03) (Fig. 1 and Table 3). Methylation of *p16* and either *CDH13*, *RASSF1A*, or *APC* in paired tumor and mediastinal lymph-node samples from the 51 case patients was associated with early recurrence (median, 9 months; range, 5 to 30), whereas in the absence of methylation of these markers, the median time to recurrence was 25 months after surgery (range, 6 to 40; P=0.04).

We examined the methylation status of *p16*, *CDH13*, *APC*, and *RASSF1A* in an independent validation cohort of 20 patients (11 case patients and 9 controls) with stage I NSCLC (Table 1). In this cohort of limited size, we validated the gene pair in the original cohort with the highest odds ratio of recurrence among case patients, as compared with controls, when methylated in both tumor and lymph nodes — *p16* and *CDH13* — using univariate analysis (Table 3 in the Supplementary Appendix). Multivariate analyses of data in the combined original and validation cohorts showed that the estimated odds of recurrence associated with methylation of *p16* and *CDH13* in tumor and mediastinal lymph nodes was 25.25 (95% CI, 2.53 to 252.35; P=0.006) for case patients as compared with controls (Table 3).

KAPLAN–MEIER ESTIMATES

Kaplan–Meier plots indicated that methylation of one or more of four genes — *p16*, *CDH13*, *RASSF1A*, and *APC* — in any sample from the patient was related to the duration of recurrence-free survival (Fig. 2A through 2D). For example, the 5-year recurrence-free survival rates for no methylated genes, one to two methylated genes, and three to four methylated genes in the mediastinal lymph nodes

Table 3. **Multivariate Odds Ratios and 95% Confidence Intervals for the Estimated Risk of Recurrence among Case Patients as Compared with Controls, According to the Methylation Status of Four Genes.***

Gene	Original Cohort (N = 167)		Original and Validation Cohorts (N = 187)	
	Odds Ratio (95% CI)	P Value	Odds Ratio (95% CI)	P Value
Unmethylated gene†	1.00		1.00	
Methylated *APC*				
Tumor	0.95 (0.45–2.04)	0.90	1.31 (0.67–2.58)	0.43
Regional nodes	0.69 (0.22–2.10)	0.51	0.78 (0.32–1.91)	0.59
Mediastinal nodes	2.26 (0.66–7.71)	0.19	1.87 (0.65–5.56)	0.25
Tumor and mediastinal nodes	2.37 (0.52–10.83)	0.27	2.00 (0.55–7.33)	0.30
Methylated *RASSF1A*				
Tumor	1.79 (0.85–3.75)	0.12	1.86 (0.94–3.68)	0.07
Regional nodes	0.71 (0.23–2.20)	0.55	0.82 (0.31–2.15)	0.68
Mediastinal nodes	1.66 (0.43–6.43)	0.46	2.13 (0.65–6.96)	0.21
Tumor and mediastinal nodes	0.66 (0.11–3.88)	0.65	0.97 (0.23–3.98)	0.96
Methylated *p16*				
Tumor	3.50 (1.65–7.41)	0.001	3.55 (1.77–7.13)	<0.001
Regional nodes	3.62 (1.41–9.32)	0.008	4.14 (1.81–9.49)	0.001
Mediastinal nodes	4.67 (1.53–14.42)	0.007	5.09 (1.96–13.18)	0.001
Tumor and mediastinal nodes	5.23 (1.33–20.46)	0.02	8.41 (2.42–29.20)	0.001
Methylated *CDH13*				
Tumor	2.12 (0.98–4.59)	0.06	2.33 (1.16–4.69)	0.02
Regional nodes	1.99 (0.81–4.88)	0.13	2.67 (1.20–5.93)	0.02
Mediastinal nodes	3.98 (1.22–13.01)	0.02	4.04 (1.53–13.63)	0.005
Tumor and mediastinal nodes	6.89 (1.36–34.87)	0.02	7.55 (1.99–28.60)	0.003
Methylated *APC* and *RASSF1A*				
Tumor	1.88 (0.74–4.78)	0.18	2.25 (1.02–5.00)	0.046
Regional nodes	1.22 (0.10–14.53)	0.88	2.34 (0.46–11.75)	0.30
Mediastinal nodes	—	—	3.49 (0.30–40.75)	0.32
Tumor and mediastinal nodes	—	—	2.37 (0.17–33.12)	0.52
Methylated *APC* and *p16*				
Tumor	4.16 (1.60–10.81)	0.004	4.48 (1.91–10.51)	0.001
Regional nodes	2.59 (0.34–19.74)	0.36	2.43 (0.59–9.94)	0.22
Mediastinal nodes	13.10 (1.20–142.73)	0.04	7.46 (1.35–41.20)	0.02
Tumor and mediastinal nodes	5.27 (0.38–73.57)	0.22	7.70 (0.70–84.88)	0.10
Methylated *APC* and *CDH13*				
Tumor	1.54 (0.55–4.31)	0.41	2.14 (0.94–4.91)	0.07
Regional nodes	2.24 (0.41–12.22)	0.35	3.30 (0.88–12.40)	0.08
Mediastinal nodes	4.58 (1.50–20.01)	0.04	3.13 (0.89–11.01)	0.08
Tumor and mediastinal nodes	11.79 (0.85–163.34)	0.07	9.48 (0.87–103.18)	0.07
Methylated *RASSF1A* and *p16*				
Tumor	5.95 (2.15–16.56)	0.001	5.26 (2.13–13.00)	<0.001
Regional nodes	1.07 (0.15–7.56)	0.94	1.92 (0.39–9.45)	0.42
Mediastinal nodes	3.01 (0.49–18.64)	0.24	4.60 (0.85–25.03)	0.08
Tumor and mediastinal nodes	2.91 (0.24–35.36)	0.40	3.67 (0.32–41.69)	0.29

Table 3. (Continued.)

Gene	Original Cohort (N=167)		Original and Validation Cohorts (N=187)	
	Odds Ratio (95% CI)	P Value	Odds Ratio (95% CI)	P Value
Methylated *RASSF1A* and *CDH13*				
Tumor	1.61 (0.67–3.89)	0.29	1.71 (0.78–3.74)	0.18
Regional nodes	0.49 (0.09–2.63)	0.41	0.88 (0.25–3.04)	0.84
Mediastinal nodes	1.36 (0.22–8.48)	0.75	1.91 (0.40–9.21)	0.42
Tumor and mediastinal nodes	2.49 (0.18–34.29)	0.50	3.51 (0.32–38.57)	0.30
Methylated *p16* and *CDH13*				
Tumor	8.00 (2.50–25.51)	<0.001	6.71 (2.50–18.00)	<0.001
Regional nodes	4.08 (1.06–15.70)	0.04	6.13 (1.99–18.89)	0.002
Mediastinal nodes	4.32 (1.06–17.65)	0.04	4.66 (1.53–14.16)	0.007
Tumor and mediastinal nodes	15.50 (1.61–185.02)	0.03	25.25 (2.53–252.35)	0.006

* Odds ratios are reported on the basis of the multivariate logistic-regression model adjusted for NSCLC stage (IA or IB), age, sex, race (white or black), histologic feature (adenocarcinoma, squamous-cell, or other), and smoking status (current or former smoker or nonsmoker). Dashes indicate that no methylation was found among samples from controls.
† This is the reference category.

were 77.3% (95% CI, 61.9 to 87.1), 51.4% (34.0 to 66.4), and 30.0% (7.1 to 57.8), respectively (P<0.001) (Fig. 2C). Among patients with stage I NSCLC, the 5-year recurrence-free survival rate in the group of patients with two or more of the four methylated genes in the primary tumor and mediastinal lymph nodes was 27.3% (95% CI, 6.5 to 53.9), as compared with 65.3% (53.1 to 75.0) in the group with fewer than two methylated genes at those sites (P<0.001) (Fig. 2D).

Methylation of both *p16* and *CDH13* in tumor and mediastinal lymph nodes was associated with a 5-year recurrence-free survival rate of 14.3% (95% CI, 0.7 to 46.5), as compared with 63.1% (95% CI, 50.2 to 73.5) in the absence of methylation of these genes (P<0.001) (Fig. 2H). This association between methylation of both *p16* and *CDH13* and survival was also found in the independent validation cohort of 20 case patients and controls (Fig. 3B).

In the validation cohort, the methylation of two or more of the four genes in tumors and mediastinal lymph nodes was associated with a lower 5-year rate of recurrence-free survival (16.7% of patients; 95% CI, 0.8 to 51.7) than that for fewer than two genes (53.8%; 95% CI, 24.8 to 76.0; P=0.04) (Fig. 3A). Similarly, methylation of *p16* and *CDH13* in tumors and mediastinal nodes resulted in a worse 5-year rate of recurrence-free survival (0.0%; 95% CI, 0.0 to 0.0) than if this pair of genes was unmethylated (53.3%; 95% CI, 26.3 to 74.4; P<0.001) (Fig. 3B).

In the original and validation cohorts combined (total, 187 patients), the methylation status of *p16* and *CDH13* was assayed in primary tumor and mediastinal lymph-node specimens from 91 patients (41 case patients and 50 controls). Of the 11 patients in whom methylation of the two genes was found in both tissue types, 10 had recurrence of the tumor within 30 months after resection, 9 within 17 months, and 8 within 12 months. Patients with two or more methylated genes in tumor and mediastinal nodes also had a worse 5-year rate of recurrence-free survival (23.5% of patients; 95% CI, 7.3 to 44.9) than those with fewer than two methylated genes (63.5%; 95% CI, 52.4 to 72.7; P<0.001) (Fig. 3C). When both *p16* and *CDH13* were methylated in the tumor and mediastinal nodes, there was a significantly lower rate of recurrence-free survival (9.1%; 95% CI, 0.5 to 33.3) than if *p16* and *CDH13* were unmethylated (61.2%; 95% CI, 49.7 to 70.9; P<0.001) (Fig. 3D).

DISCUSSION

Our study indicates that methylation of the promoter regions of certain genes in a resected NSCLC specimen is associated with recurrence of the tumor. A relation between gene methylation and tumor recurrence has been shown.[29,30] The four genes of interest in our study are *p16*, *CDH13*, *APC*, and *RASSF1A*. They are involved in cell-cycle control (*p16*), invasion and metastasis (*CDH13*, *APC*), and *Ras* signaling (*RASSF1A*). Other studies of *p16*

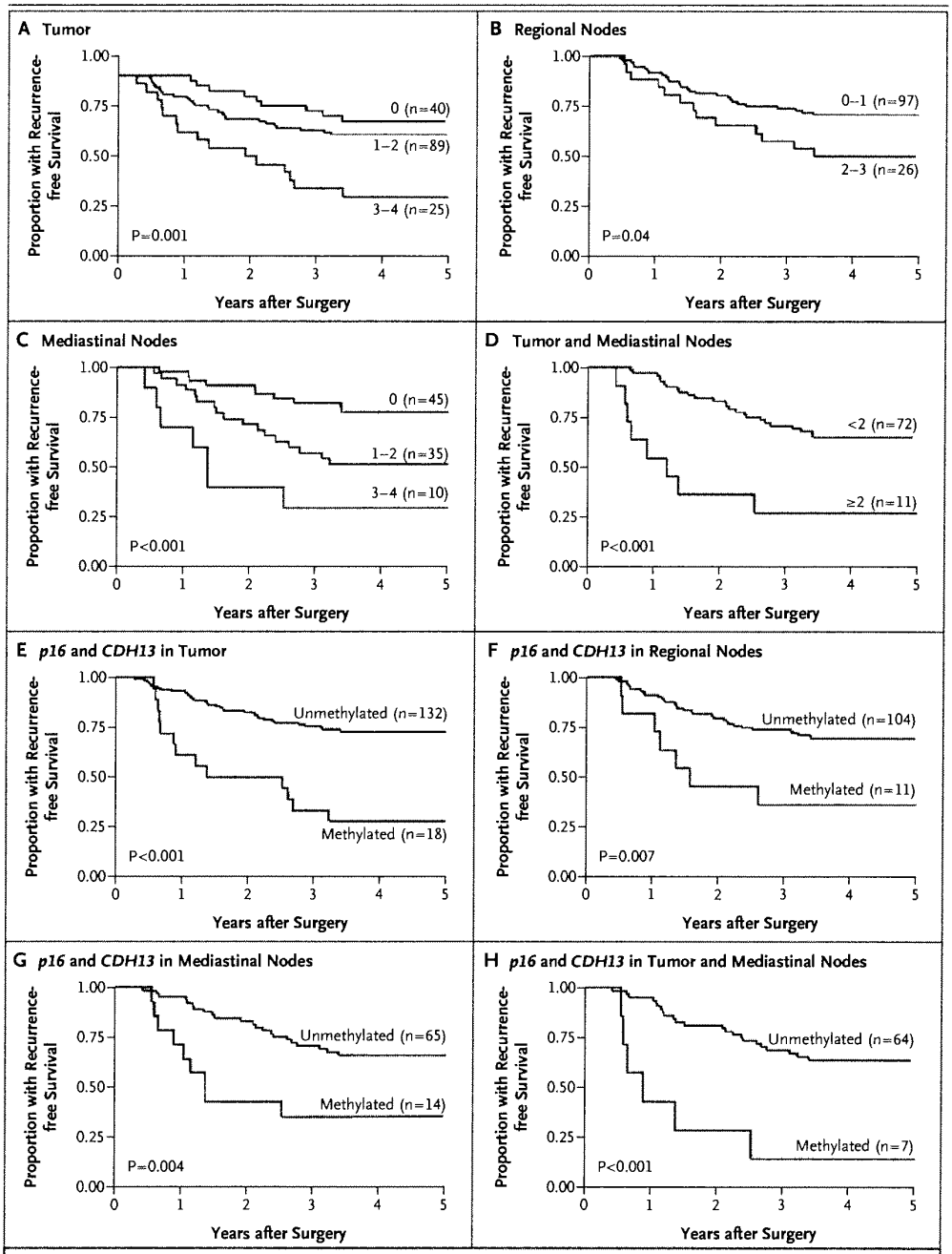

Figure 2. **Kaplan–Meier Estimates of Recurrence-free Survival among 167 Case Patients and Controls with Stage I Non–Small-Cell Lung Cancer from the Original Cohort, According to the Site and Number or Presence or Absence of Methylated Genes.**

Data are reported for a four-gene panel consisting of the cyclin-dependent kinase inhibitor 2A gene *p16*, the H-cadherin gene *CDH13*, the *Ras* association domain family 1 gene *RASSF1A*, and the adenomatous polyposis coli gene *APC*. In all three types of tissue, the recurrence-free survival rates decrease with an increasing number of genes (Panels A, B, and C) and when the genes are methylated (Panels E, F, and G). This same effect on recurrence-free survival is evident when the tumor and mediastinal lymph nodes are considered together (Panels D and H). The numbers of patients vary among panels because not every sample was informative for every gene.

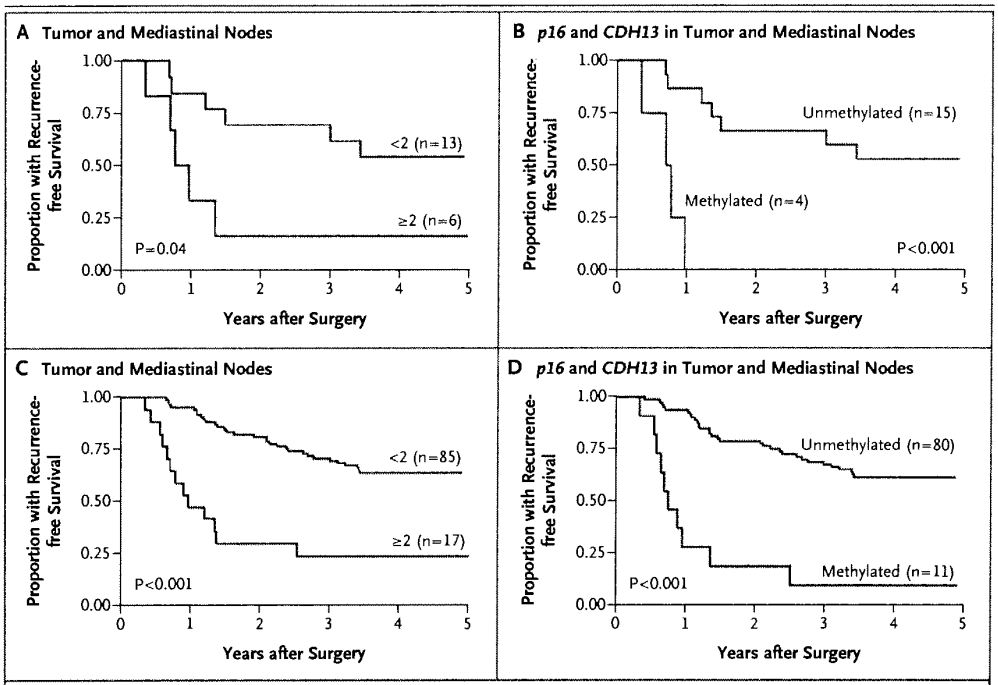

Figure 3. Kaplan–Meier Estimates of Recurrence-free Survival in the Validation Cohort or the Validation and Original Cohorts, According to the Site and Number or Presence or Absence of Methylated Genes.

Data are reported for a four-gene panel consisting of the cyclin-dependent kinase inhibitor 2A gene *p16*, the H-cadherin gene *CDH13*, the *Ras* association domain family 1 gene *RASSF1A*, and the adenomatous polyposis coli gene *APC*. Panels A and B show data for the independent validation cohort of 20 case patients and controls. Panels C and D show data for the combined original and validation cohorts (total, 187 patients). The numbers of patients vary among panels because not every sample was informative for every gene.

expression or promoter-region methylation in lung cancer have focused mostly on the primary tumors,[10,13,18,31-33] but we found that molecular examination of lymph nodes improves the assessment of risk of recurrence. The methylation of these genes in histologically normal regional lymph nodes probably indicates the presence of microscopically undetectable micrometastases. Immunohistochemical analyses, for example, may miss a rare cell in the background of normal tissue, whereas the methylation-specific PCR assay is sufficiently sensitive to detect a signal of DNA methylation.

The current method of assessment of the risk of recurrence in patients with stage I (T1–2N0) NSCLC is imprecise — one third of such tumors recur after curative surgery. Our results suggest that the detection of promoter methylation of certain genes may identify cells with a potential for metastatic spread not only within NSCLC but also in lymph nodes. It is possible that the methylated genes in lymph nodes represent tumor DNA that drained to the nodes through the lymphatic system, but this is unlikely because mediastinal nodes are located far from the lung, in the mediastinum. The correlation between short survival and the number of methylated genes in the regional and mediastinal lymph nodes supports the presence of micrometastases in those sites. Recent promising results for predicting the risk of lung cancer[34] or its recurrence[17] have been obtained by examining changes in gene methylation in sputum.[34] Our study was retrospective and conducted at a single institution, and the number of patients studied was small. For these reasons, replication of our findings in a large, prospectively studied cohort is essential before the four-gene panel we investigated can be used in clinical practice.

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RESEARCH ARTICLES

5-Azacytidine Hydrolysis Kinetics Measured by High-Pressure Liquid Chromatography and ^{13}C-NMR Spectroscopy

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Abstract □ Hydrolysis of 5-azacytidine, an experimental anticancer drug, in aqueous buffers was measured using a high-pressure liquid chromatographic (HPLC) procedure and a ^{13}C-NMR method. The former utilized a 17.5-μm Aminex A-6 strong cation-exchanger column eluted with 0.4 *M*, pH 4.6 ammonium formate buffer at a flow rate of 0.4 ml/min. The hydrolysis sequence as well as the existence of a labile intermediate, *N*-formylguanylribosylurea, was unequivocally established using 6-^{13}C-5-azacytidine and NMR spectral techniques. A reversible ring opening step to the *N*-formylguanylribosylurea with an equilibrium constant of 0.58 ± 0.03 between pH 5.6 and 8.5, followed by an irreversible formation of guanylribosylurea, was found by HPLC. The data confirm previous assumptions on the hydrolytic kinetics. The pH dependency of hydrolysis was examined, and the hydrolysis profile gave a normal V shape with the most stable pH at 7.0. Rather stable intravenous dosage forms can be formulated.

Keyphrases □ 5-Azacytidine—hydrolysis kinetics, aqueous solutions, high-pressure liquid chromatography, ^{13}C-NMR □ Hydrolysis kinetics—5-azacytidine, aqueous solutions □ Antineoplastic agents—5-azacytidine, hydrolysis kinetics, aqueous solutions

A cytidine analog, 5-azacytidine[1] (I), first synthesized in 1964 (1) and subsequently isolated from *Streptoverticillium ladakanus* (2, 3), has shown antitumor activity against several animal neoplasms including L-1210 leukemia (4) and T-4 lymphoma (5). Clinically, it has also demonstrated activity against various solid tumors (6) as well as leukemias (7) and is currently undergoing phase II trials (8).

This compound has long been known to be unstable in aqueous solution. Its lability has been attributed to the facile hydrolytic cleavage across the 5,6-bond (9–11). Consequently, proper formulation has been a problem in its clinical use. In addition, not only has the metabolic evidence indicated that ring cleavage is a major process of its disposition (12–14), the facile process may have a possible relationship to its still unclear overall biological activity. Therefore, the nature and sequence of its hydrolysis must be understood. While hydrolysis of I has been investigated (9–11, 15), the lack of a suitable analytical method has hampered a complete and unequivocal kinetic analysis, although certain information concerning hydrolysis has been obtained through spectroscopic resolution in a complex hydrolytic mixture (10).

Recently, specific information was obtained through TLC, NMR (11), and high-pressure liquid chromatography (HPLC) (15), including the isolation and identification of the labile intermediate; however, a systematic kinetic analysis is still lacking. This paper describes an HPLC method for the simultaneous analysis of I and its labile intermediate, *N*-formylribosylguanylurea (II), which occurs during formation of the hydrolytic product 1-β-D-ribofuranosyl-3-guanylurea (III). Corroborated with Fourier transform ^{13}C-NMR, using a 6-^{13}C-5-azacytidine previously synthesized in this laboratory (14), a detailed kinetic analysis of the hydrolysis of I in aqueous solution is presented.

EXPERIMENTAL

Chemicals and Reagents—All solvents were either analytical or liquid chromatographic grade. 5-Azacytidine was greater than 99% pure by HPLC and was used without further purification. Ninety percent labeled 6-^{13}C-5-azacytidine was synthesized as described previously (14). 1-β-D-Ribofuranosyl-3-guanylurea (III) was synthesized according to

[1] 4-Amino-1-β-D-ribofuranosyl-1,3,5-triazin-2-one, NSC-102816, CAS Reg. No. 320-67-2. Supplied by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD 20014.

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the procedure of Pithova *et al.* (9). Sodium formate[2] and 90% enriched 1-^{13}C-sodium acetate[3], used as internal standards, were also pure by ^{13}C-NMR.

Analytical Procedure—Methanolic or aqueous solutions of 5-azacytidine were injected into a liquid chromatograph[4,5] *via* a 20-μl high-pressure sample injection valve[5]. The solution was eluted into a 500 × 2-mm i.d. stainless steel column[5] packed with 17.5-μm Aminex A-6[6] strong cation-exchanger with 0.40 *M*, pH 4.6 ammonium formate buffer at a flow rate of 0.4 ml/min. The components emerging from the column were detected *via* a UV detector[5] set at 254 nm. The UV-absorbing components were quantitated using either peak height or peak area as estimated *via* a mechanical disk integrator on a strip-chart recorder[7]. No significant difference was observed between these two quantitative methods.

^{13}C-NMR—NMR studies of 5-azacytidine, 6-^{13}C-5-azacytidine, and guanylribosylurea were recorded in dimethyl sulfoxide or in aqueous buffers at pH 8.0 *via* a 25.2-MHz Fourier transform NMR spectrometer[8]. Typical parameters for the measurements were: acquisition time, 0.8 sec; pulse delay, 1.2 sec; sweep width, 5000 Hz, 8 K data points; and tip angle, 30°.

For kinetic measurements, an aqueous solution of 90% enriched 6-^{13}C-5-azacytidine, 0.1 *M* in pH 8.0, 0.067 *M* phosphate buffer, was placed in the sample tube, which was inserted into the spectrometer probe. The probe temperature was maintained at 38 ± 0.1°. Rapid pulses at a pulse width of 8 were generated, and the appropriate number of transients was accumulated to acquire an adequate signal-to-noise ratio. A timer was started when the 5-azacytidine solution was mixed and placed inside the spectrometer. At the end of each accumulation, the time was noted.

Kinetic Measurements—The hydrolytic degradation of 5-azacytidine was studied quantitatively by HPLC at 25 and 37° in 0.067 *M* sodium phosphate buffer and acetate buffer at pH 4.5–9.1.

All experiments were duplicated. A gas-tight syringe was filled with a solution of 5-azacytidine at either 4.10×10^{-4} or 8.20×10^{-4} *M* in a phosphate buffer of the desired pH. For the 25° runs, the filled syringe was allowed to remain at room temperature in the high-pressure injector valve module. At approximately 10-min intervals, aliquots were pushed into the sample loop and injected immediately. For the 37° runs, the syringe was filled, capped, and placed in a constant-temperature bath. It was then removed at specific intervals to fill the injector loop and quickly replaced in the bath.

The 5-azacytidine concentrations were followed by either the peak height or the peak area method. Stability evaluations were made in Ringer's lactate and in normal saline at room temperature as well as at refrigerated temperature (5°).

RESULTS

Drug Assay—With either methanolic or aqueous solutions of 5-azacytidine kept at ice temperature before injection, a straight line was observed in the $0.041–8.20 \times 10^{-4}$ *M* range when either peak heights or peak areas were plotted against concentrations. The variance of 10 separate injections at 0.20×10^{-4} *M* was less than 1%.

When 5-azacytidine was dissolved in a phosphate buffer, 0.067 *M* at pH 8.0, and chromatographed, in addition to a peak emerging at 3.4 min (peak 1) corresponding to 5-azacytidine, a small, slower running peak emerged at 7.3 min (peak 2) and the intensity of peak 2 appeared to increase with time (Fig. 1). Their peak height ratio (peak 1 to peak 2) appeared to be constant at approximately 3:2 after 150 min. The intensities of these two peaks then diminished with time while maintaining an essentially constant ratio.

Each component was collected into a separate tube at room temperature. Reinjection of peak 1 immediately following collection yielded a single peak corresponding to 5-azacytidine. However, similar treatment of peak 2 gave two components corresponding to peaks 1 and 2, the latter at a higher intensity. After a longer period, repetition of the injection of either peak 1 or 2 revealed two peaks, this time with a peak 1 to peak 2 ratio of approximately 3:2 but with both at much diminished intensities. Collection of peak 2 at ice temperature did not appear to change the reversal of peak 2 to peak 1.

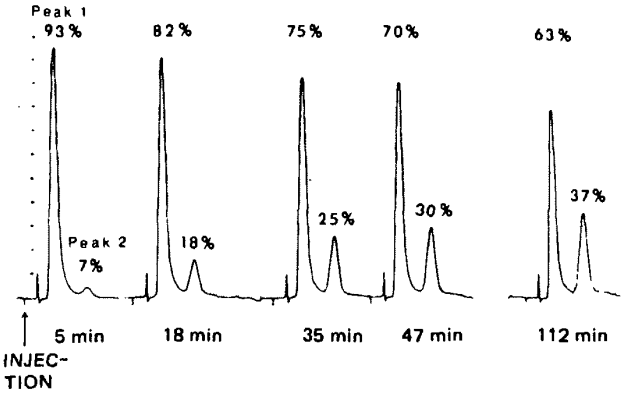


Figure 1—*High-pressure liquid chromatograms of 5-azacytidine, 8.2 × 10^{-4} M in pH 8.0, 0.067 M potassium phosphate at 25°, using 0.4 M, pH 4.6 ammonium formate at 0.4 ml/min.*

Attempts to isolate peak 2 by collecting it into a tube in a dry ice–acetone bath followed by lyophilization failed[9]. Dissolving the residue in water and injecting it into the liquid chromatograph did not give significant amounts of a UV-absorbing peak (254 nm). TLC analysis of the residue using the literature procedure (14) indicated that guanylribosylurea (III) was a major component.

^{13}C-NMR Analysis—The naturally abundant proton-decoupled NMR spectra of 5-azacytidine and guanylribosylurea spiked with formate in aqueous buffer are shown in Figs. 2 and 3, respectively. The chemical shift assignments of 5-azacytidine under the present conditions were referenced from reported values (16), and those for guanylribosylurea were assigned by direct comparison with those of I (Table I). Differences of the chemical shifts of guanylribosylurea from 5-azacytidine were seen in C-2, C-4, and C-1, as expected from the structure.

When a 5-azacytidine solution in pH 8 phosphate buffer was subjected to Fourier transform NMR analysis, new sets of signals not accounted for on the basis of the 5-azacytidine and ribosylguanylurea spectra appeared with time. A new signal appeared at 171.7 ppm, subsequently identified as formate by signal enhancement with the addition of sodium formate. Furthermore, the signal at 157.5 ppm was particularly broadened (Fig. 4).

The kinetic detections of 6-^{13}C-5-azacytidine hydrolysis in a buffered solution are shown in Fig. 5. A known concentration of ^{13}C-sodium acetate was added to the buffered solution in an attempt to quantitate the decomposition kinetics. The signals ratio between the C-6 of 5-azacytidine and the C-1 of sodium acetate was essentially constant until after 17 min. A small signal appeared at 167.8 ppm and increased with time. After 90 min, a second signal at 171.7 ppm appeared. Both new signals increased at the expense of the C-6 of 5-azacytidine, as indicated by slowly decreasing ratios of the signal intensity of the C-6 of 5-azacytidine to the C-1 of acetate. After 270 min, the intensity of the lower field signal exceeded that of the higher field one. The time course of the ratios between the C-6 signal of 5-azacytidine and the C-1 of acetate, an added internal standard, is shown in Fig. 6.

The identity of these new signals was confirmed by off-resonance decoupled NMR measurements, which assess the ^{13}C–H coupling. In this experiment, all signals exhibited a doublet except the signal of acetate (Fig. 5*h*), which indicated the attachment of one proton to each carbon. The chemical shift of the signal at 171.7 ppm coincided with that of the formate. On the basis of the chemical rationale of hydrolysis, UV absorption property *via* HPLC, and C–H coupling *via* the NMR off-resonance decoupling experiments, the signal at 167.8 ppm was assigned as derived from the formyl carbon of the labile intermediate, *N*-formylguanylribosylurea.

Kinetic Analysis—5-Azacytidine hydrolysis was carried out in 0.067 *M* phosphate buffers at pH 4.5–9.1 and at 25 and 37°. Selected hydrolysis profiles as measured by concentrations of 5-azacytidine *versus* times are shown in Fig. 7. Either monophasic or biphasic profiles were observed in all of the pH and temperature studies on semilog plots. At low pH as well as in the vicinity of neutral pH, biphasic profiles were observed, with

[2] Mallinckrodt.
[3] Merck & Co., St. Louis, Mo.
[4] Chromatronix model 3510.
[5] Spectra-Physics, Santa Clara, Calif.
[6] Packed in this laboratory with the resin supplied by Bio-Rad Laboratories, Richmond, Calif.
[7] Varian Aerograph, model 20, Varian Associates, Palo Alto, Calif.
[8] Varian XL-100, Varian Associates, Palo Alto, Calif.

[9] Recently, Beisler (11) was able to isolate and characterize *N*-formylribosylguanylurea by HPLC using a C-18 reversed-phase column and water as the eluant. In the present case, the low pH and high salt content during the lyophilization procedure may have caused the decomposition of this labile intermediate.

Table I—Chemical Shifts[a] of 5-Azacytidine and Guanylribosylurea

Carbon	I	III
C-4	166.4	162.2
C-6	157.5	—
C-2	156.1	165.4
C-1′	91.8	85.5
C-4′	84.6	83.9
C-3′	74.9	74.3
C-2′	69.6	71.2
C-5′	61.1	62.7

[a] Measured in dimethyl sulfoxide and expressed in parts per million with tetramethylsilane as the internal standard.

the most pronounced being at pH 7 and 8. At high pH, *i.e.*, 9.1, the first phase was too rapid to be discernible so that an apparently monophasic profile was observed. Similar biphasic profiles of 5-azacytidine were observed in Ringer's lactate as well as in normal saline, although the terminal phases declined very slowly.

From this evidence for the existence of an initial equilibrium phase between *N*-formylguanylribosylurea and 5-azacytidine, the biphasic behavior of these hydrolysis profiles were attributed to the kinetic scheme of Pithova *et al.* (9) (Path A, Scheme I).

The appropriate rate constants can be solved by making several assumptions: (*a*) the initial process is a rapid equilibrium, (*b*) *N*-formylguanylribosylurea exists at an approximate steady state throughout the hydrolysis, and (*c*) $k_{-1} \gg k_2$. These assumptions were supported by subsequent estimated data, generating a self-consistency. Then k_1 and k_{-1} are solvable by:

$$K_{eq} = \frac{k_1}{k_{-1}} \qquad \text{(Eq. 1)}$$

and (17, 18):

$$\log\left(\frac{A_0 - A_{eq}}{A - A_{eq}}\right) = \frac{k_1 + k_{-1}}{2.303} t \qquad \text{(Eq. 2)}$$

k_2 is solvable by the first-order degradation of the second phase:

$$A = A_0 e^{-Kt} \qquad \text{(Eq. 3)}$$

$$K = K_{eq} k_2 \qquad \text{(Eq. 4)}$$

The estimated rate constants for 5-azacytidine hydrolysis in aqueous buffer solutions at different pH values and at two temperatures are shown in Table II. The stability profile at 25 and 37° as expressed by plotting log k_1 *versus* pH is shown in Fig. 8. The profile at both temperatures followed a typical V shape, with the most stable pH at 7.0.

DISCUSSION

As was first proposed by Pithova *et al.* (9), 5-azacytidine in aqueous solution undergoes hydrolysis according to Scheme I in strong aqueous acid. Glycosidic linkage cleavage to yield 5-azacytosine, 5-azauracil, and D-ribose (Path B) was the major degradative pathway. In neutral and basic media, a facile ring cleavage to yield an instable *N*-formylguanylribosylurea intermediate followed by a loss of formate to form ribosylguanylurea was proposed as the major degradative pathway (Path A). Notari and DeYoung (10) proposed a refined scheme to include the hydration step across the 5,6-double bond as the first step.

In those studies, UV spectral techniques were used to quantify the hydrolysis kinetics of I. Using a TLC and PMR method, Israili *et al.* (15) studied the hydrolysis of 5-azacytidine and found that its degradation in aqueous buffers as well as in human plasma under various conditions followed first-order kinetics, but only monoexponential declines were reported.

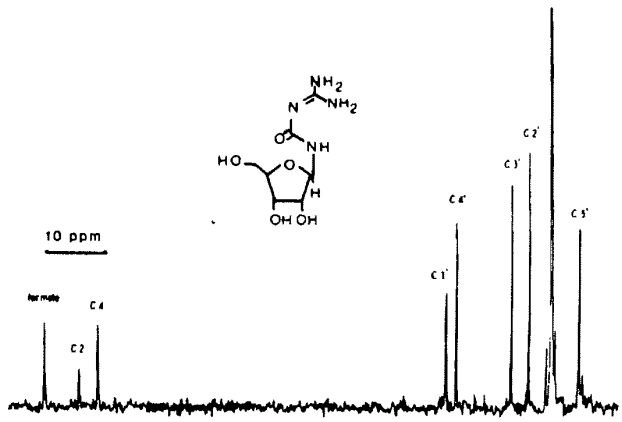
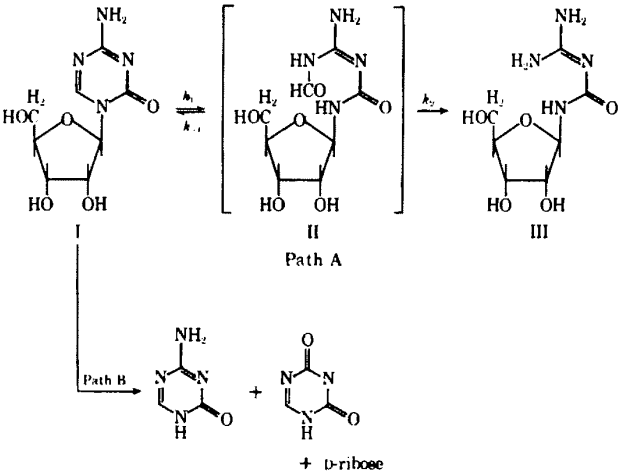


Figure 3—^{13}C-*NMR spectrum of guanylribosylurea (0.15 M) and sodium formate (0.32 M) in deuterium oxide–water at pH 10.2 and 30°.*



Scheme I—Proposed hydrolysis reactions of 5-azacytidine after Pithova et al. (9). Path A is in aqueous buffers, and Path B is in strong acids.

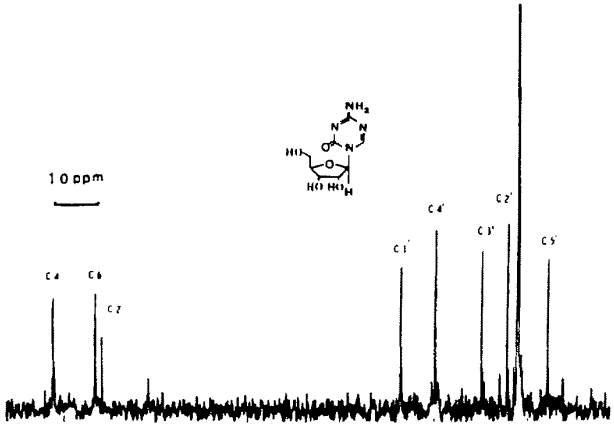


Figure 2—^{13}C-*NMR spectrum of 0.15 M 5-azacytidine at 30°. The solvent was deuterium oxide–water.*

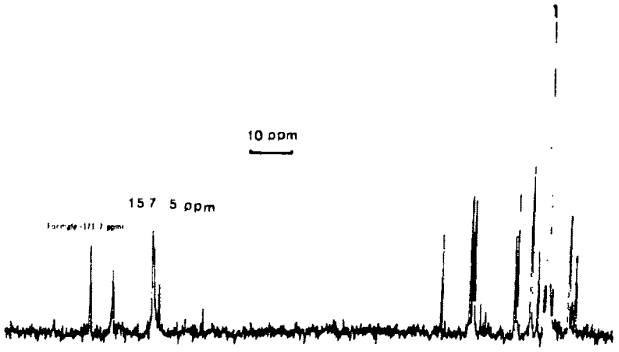


Figure 4—^{13}C-*NMR spectrum of 5-azacytidine in pH 8.0 phosphate buffer (0.15 M) after 4 hr at 30°.*

Table II—Estimated Rate Constants[a] for 5-Azacytidine Hydrolysis in Aqueous Buffer Solutions

pH	25° $k_1 \times 10^{-3}$ M, min^{-1}	25° $k_{-1} \times 10^{-3}$ M, min^{-1}	25° K_{eq}	25° $k_2 \times 10^{-3}$ M, min^{-1}	30° $k_1 \times 10^{-3}$ M, min^{-1}	30° $k_{-1} \times 10^{-3}$ M, min^{-1}	30° K_{eq}
4.5	7.67 ± 0.37	17.50 ± 0.40	0.44	4.10	15.60 ± 0.40	32.30 ± 1.40	0.48
5.6	6.27 ± 0.37	11.20 ± 1.20	0.57	1.30	11.20 ± 0.60	19.60 ± 1.50	0.58
7.0	3.22 ± 0.02	5.73 ± 0.04	0.56	0.77	7.33 ± 0.00	13.05 ± 0.00	0.56
7.4	4.36 ± 0.04	7.95 ± 0.24	0.55	0.93	12.8 ± 0.50	22.20 ± 0.50	0.58
8.0	9.92 ± 0.03	17.20 ± 0.50	0.58	1.70	35.00 ± 1.80	60.80 ± 1.70	0.58
8.5	24.00 ± 1.10	39.20 ± 1.8	0.61	—	—	—	—
9.1	62.20 ± 3.80	101.0 ± 6.0	0.61	10.80	—	—	—

[a] Calculations were based on the molar concentrations of remaining 5-azacytidine since the *N*-formyl intermediate possesses different extinction coefficients (10, 11). At those times where initial equilibrium was observed and used for calculation, insignificant amounts of irreversible degradation occurred, consistent with the assumptions made, *i.e.*, $k_{-1} \gg k_2$, and with the experimental observations (^{13}C-NMR). This finding is also consistent with the observations in Ref. 11.

None of the previous studies isolated and characterized the *N*-formylguanylribosylurea intermediate. In a preliminary study (19) in this laboratory, the *N*-formyl intermediate was isolated and characterized by ^{13}C-NMR. Recently, using HPLC and PMR, Beisler (11) also isolated and characterized the *N*-formyl intermediate and reported its biological activity. However, no detailed kinetic analysis of the hydrolysis of I was presented.

The aims of this study were to use ^{13}C-NMR coupled with specific stable isotopic labeling to demonstrate unequivocally the existence of the unstable intermediates and the sequence of the degradation and to use an assay method to quantitate the kinetics and hydrolysis of 5-azacytidine as a function of pH and temperature.

^{13}C-NMR—Because of the high sensitivity and speed in data acquisition, Fourier transform ^{13}C-NMR has been used in studying reaction mechanisms (20, 21). In the present studies, the large differences in chemical shifts among the imino carbon of I, the *N*-formyl carbon of II, and the formic acid carbon were utilized for analysis. The C–H coupling (*i.e.*, doublet) should provide a positive identification of their location. Initially, it was anticipated that NMR studies on the basis of naturally abundant C-13 would provide the necessary evidence for the hydrolysis; however, no additional signals at the low field region except the formate carbon have been revealed in the hydrolysate. Subsequently, it was found that, due to the overlapping signal between the C-6 of *N*-formyl carbon and the C-4 and 5-azacytidine, it was not possible to follow the kinetics of the generation of II using naturally abundant ^{13}C-compounds. This problem was circumvented by the use of ^{13}C-5-azacytidine labeled at the C-6 position synthesized previously (14).

Use of 90% labeled 6-^{13}C-5-azacytidine revealed the generation of *N*-formylribosylguanylurea and subsequently formic acid from a solution of 5-azacytidine in pH 8.0 phosphate buffer. The chemical shifts and multiplicity in off-resonance decoupled ^{13}C-NMR and UV absorption characteristics detected from the HPLC studies are all consistent with the assigned *N*-formylguanylribosylurea structure. In addition to the off-resonance decoupled experiment, the formate carbon identity comes from enhancement of signal intensity with addition of sodium formate. On the basis of the timed ^{13}C-NMR studies, the generation sequence of *N*-formylguanylribosylurea and formate was proven unequivocally. Although carbinolamine formation as the first step of hydration across the 5,6-double bond (10) appeared to be reasonable, its detection by ^{13}C-NMR has not yet been accomplished, possibly because of its short life or very low concentration. Thus, this study also demonstrates the use of stable isotope labeling and ^{13}C-NMR in the study of reaction mechanisms.

Since the relaxation mechanism and T_1 of 5-azacytidine and *N*-formylguanylribosylurea are likely to be similar, it was thought that by selecting a reference atom with a similar chemical shift, the degradation kinetics could be followed. Thus, 90% 1-^{13}C-sodium acetate equimolar to 6-^{13}C-5-azacytidine was added to the solution for the kinetic measurement. Decomposition kinetics were followed by a change in the signal ratios between the C-6 of 5-azacytidine and the C-1 of acetate (Fig. 6). However, as shown, the ratio remained relatively constant for the initial several minutes and declined afterwards. The kinetic profile was not consistent with the HPLC data and remained difficult to interpret. The difference was perhaps due to in part to errors in Fourier transform NMR arising from limitations because of the number of data points available in the computer system and from the nonuniformity in numbers of pulses for each time spent. Therefore, steady state may not have been achieved in the experiments with few pulses.

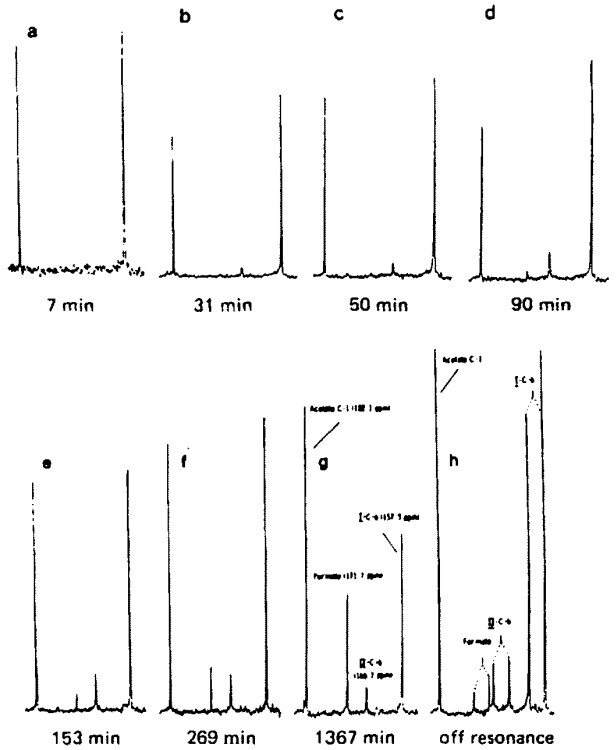


Figure 5—*^{13}C-NMR spectrum of 90% enriched 6-^{13}C-5-azacytidine (0.148 M) in pH 8.0, 0.067 M potassium phosphate buffer containing 30% deuterium oxide and ^{13}C-sodium acetate (0.20 M) at 27°. Figures 5a–5h are spectra at selected times. For complete number of time points, see Fig. 6.*

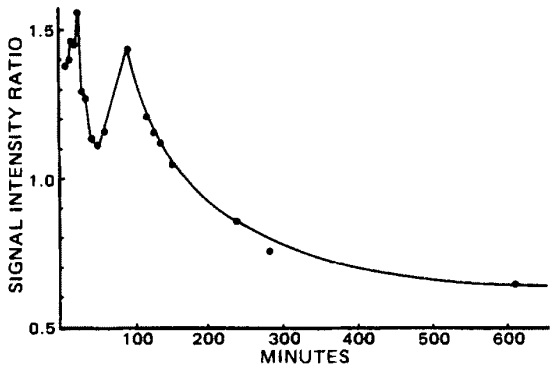


Figure 6—*Kinetic profiles of 90% enriched 6-^{13}C-5-azacytidine (0.158 M) hydrolysis in pH 8.0, 0.067 M potassium phosphate buffer containing 30% deuterium oxide and ^{13}C-sodium acetate (0.20 M) at 27° using ^{13}C-NMR measurements. Vertical axis is signal intensity ratios between 5-azacytidine and sodium acetate.*

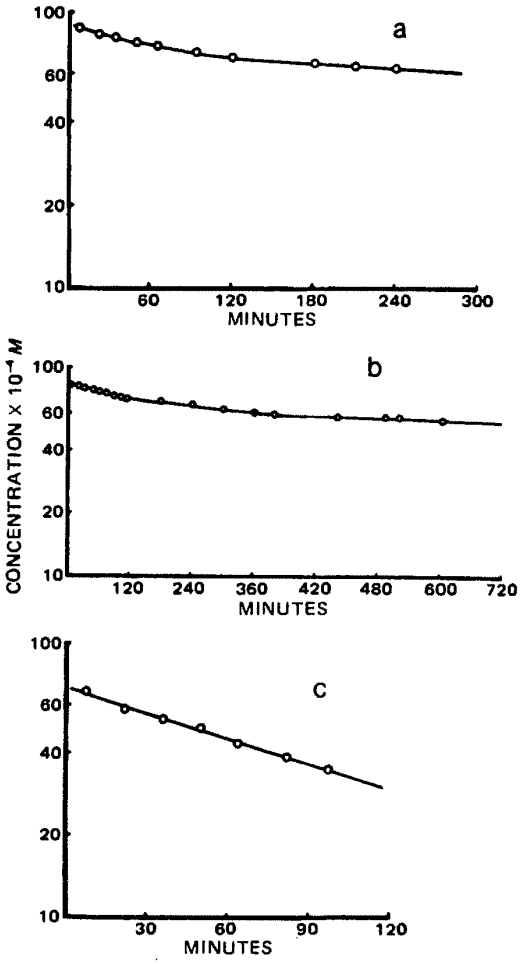


Figure 7—*Kinetic profiles of 5-azacytidine (8.2 × 10^{-4} M) hydrolysis in potassium phosphate buffers using HPLC measurements. Key:* (a), *pH 5.6 and 2.5°;* (b), *pH 7.0 and 25°; and* (c), *pH 9.1 and 25°.*

Kinetics Studies—Using UV measurement at 242 nm, Pithova *et al.* (9) first observed an initial increase followed by a subsequent decline when the extinction coefficient of 5-azacytidine in pH 6.6–7.8 borate buffers was plotted against time. Using similar UV measurements at two different wavelengths, Notari and De Young (10) observed biphasic profiles when 5-azacytidine absorbances in buffer solutions were plotted against time; the decrease or increase with time of the initial phase depended on whether the sample was acidified initially or not. The initial phase decreased with time using initially acidified sample and increased using nonacidified 5-azacytidine.

In the present study (Fig. 7), in buffers from pH 4.5 to 8.5, biphasic declines were observed when 5-azacytidine concentrations were plotted against time. At pH 9.1, the profile appeared to be monophasic and probably resulted from a very rapid equilibrium so that the first phase was not readily discernible. On the basis of the kinetic scheme (Scheme I), the individual rate constants were estimated (Table II). The direct kinetic measurements showed a rapid equilibrium between 5-azacytidine and *N*-formylribosylguanylurea, followed by a slower degradation of the latter to ribosylguanylurea.

As was evident at room temperature and various pH values, the rate constants, k_{-1}, for the ring closure were several times larger than those of the guanylribosylurea formation, k_2, consistent with the assumption made. This observation is reasonable in view of the chemical nature of the cleavages since the latter step requires the breakage of an amide bond. The k_{-1} values were also slightly greater than the k_2 values, the rate constant for the ring opening, yielding an equilibrium constant of 0.58 ± 0.03 between pH 5.6 and 8.5. This value is close to the constant ratio of 0.41 between II and I reported in water (pH unknown) (11). At 37° the equilibrium constants (0.58 ± 0.01) remained essentially unchanged (pH 5.6–8.0). The equilibrium constant decreased slightly at a lower pH (4.5). These observations are qualitatively consistent with the HPLC and PMR observations as well as with reported data (10, 11).

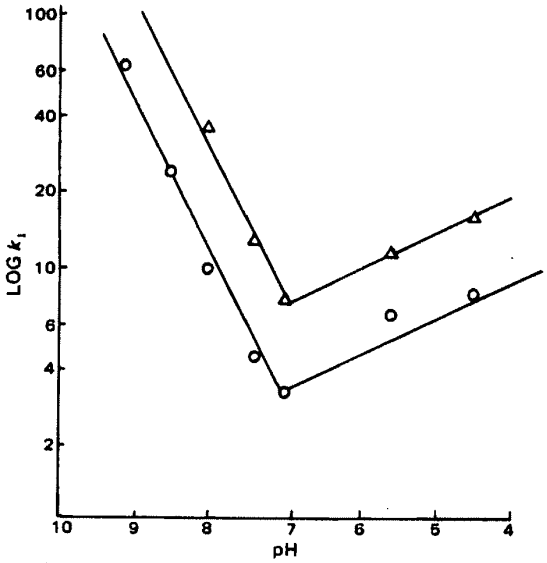


Figure 8—*The pH stability profile of 5-azacytidine (8.2 × 10^{-4} M) in aqueous buffers (potassium phosphate buffer, 0.067 M) at 25° (○) and 37° (△).*

pH-Dependent Hydrolysis—As shown in Table II, the rate constants of the hydrolysis varied with pH. When log k values were plotted against pH at 25 and 37°, a V-shaped curve resulted with both minima at pH 7.0 (Fig. 8), a value slightly different than the pH 6.5 reported (10). The slopes on the basic and acidic portions of the curve were +0.630 and −0.152, respectively. A similar trend was also observed if log k_2 values were plotted against pH. The hydrolysis, therefore, appears to be acid and base catalyzed, similar to the other known examples (22, 23). The deviation of the slopes from +1 and −1 on the basic and acidic pH portions of the curve, respectively, suggests that the hydrolysis may be susceptible to general acid and base catalyses (17). These pH profiles of the hydrolyses were markedly different from those reported by Notari and De Young (10) who described a more complex profile not readily interpretable on the basis of acid and base catalyses.

The hydrolysis kinetics observed were qualitatively similar to those reported using HPLC, PMR, and UV; however, quantitation differences were observed with those reported and may be attributed to differences in experimental conditions as well as methodologies. Under comparable experimental conditions such as those in Ringer's lactate, comparable results were obtained.

Stability Evaluation of 5-Azacytidine in Aqueous Buffers and Other Solutions—Although 5-azacytidine hydrolysis is subject to acid and base catalyses, the rate constants for the reversible formation of *N*-formlyribosylguanylurea as well as for the irreversible formation of ribosylguanylurea are smaller in magnitude than at the basic pH. Since ribosylguanylurea itself does not seem to possess significant cytotoxicity, rapid degradation of 5-azacytidine to this compound will result in a loss of efficacy. Recent biological data suggest that *N*-formylribosylguanylurea also has little cytotoxicity (11), although the evidence was not clearcut. However, other researchers found that aqueous solutions of 5-azacytidine, after long standing at room temperature, resulted in higher cytotoxicity (24, 25). Although formation of *N*-formylribosylguanylurea is reversible, in the absence of more definitive data it is desirable to formulate 5-azacytidine in media where the ring opening reaction is retarded. The pH profile of hydrolysis and kinetic data in Table II show that the rate constants for the hydrolysis in acidic pH are smaller in magnitude than those in basic pH. Therefore, a pH lower than 7.0 will result in slower ring hydrolysis.

In patients, slow infusion of 5-azacytidine in Ringer's lactate has lowered GI toxicity. The present study demonstrated that 5-azacytidine is very stable in this medium (pH 6.4), yielding a terminal $t_{1/2}$ of 4.8 days at room temperature, a value close to the reported one of 4.2 days (15). The $t_{1/2}$ in this medium at refrigerated temperature (0–5°) was considerably longer (31.3 days).

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GI Absorption of β-Lactam Antibiotics III: Kinetic Evidence for *In Situ* Absorption of Ionized Species of Monobasic Penicillins and Cefazolin from the Rat Small Intestine and Structure–Absorption Rate Relationships

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Received July 6, 1978, from the *Faculty of Pharmaceutical Sciences, Kanazawa University, Takara-machi, Kanazawa 920, Japan.* Accepted for puhlication December 20, 1978.

Abstract □ Absorption rates of monobasic β-lactam antibiotics were measured as a function of lumen solution pH between 4 and 9 by utilizing the rat intestinal recirculating method *in situ*. Between pH 6.5 and 9, the absorption rate constants of ionized antihiotics were almost identical; but, at pH 4, the unionized species were highly absorbed, depending on their lipophilicity through the GI membrane lipoidal barrier. The structure -absorption rate relationship was estahlished with the unstirred layer model.

Keyphrases □ Absorption, GI—various monobasic penicillins and cefazolin, effect of ionization, pH, structure–activity relationships □ Penicillins, various—GI absorption, effect of ionization, structure–activity relationships □ Cefazolin—GI absorption, effect of ionization, structure–activity relationships □ Antibiotics—penicillins and cefazolin, GI absorption, effect of ionization, structure–activity relationships

The GI absorption rate of a monobasic penicillin in rats deviated significantly from the pH-partition hypothesis (1). This shift was interpreted successfully by the absorption mechanism of penicillins through the aqueous diffusion layer of the lumen side of the GI membrane, which is restricted by the lipophilicity of the undissociated species (1).

In *in vitro* experiments utilizing an isolated gut technique, the transport of phenoxypenicillin derivatives exhibited a saturable process, but additional study did not produce evidence of active transport (2). A similar *in vitro* study (3) provided evidence for the passive transport of β-lactam antibiotics at pH 7.4. Perrier and Gibaldi (4) attributed the larger absorption of dicloxacillin than of penicillin G and ampicillin from the *in situ* rat intestinal loop to the larger lipophilicity of dicloxacillin. A good correlation was found (5) between the rate of *in situ* intestinal cephalexin and cefazolin absorption in rats and their *in vitro* release rate from liposomes.

Recent studies in this laboratory on the *in situ* absorption kinetics of low lipophilic and completely ionized amphoteric β-lactam antibiotics, such as amoxicillin (6), cyclacillin (7), and cephradine (7), indicated that their intestinal absorption is governed by simple diffusion fol-

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5-Azacytidine and 5-aza-2′-deoxycytidine as inhibitors of DNA methylation: mechanistic studies and their implications for cancer therapy

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5-Azacytidine was first synthesized almost 40 years ago. It was demonstrated to have a wide range of anti-metabolic activities when tested against cultured cancer cells and to be an effective chemotherapeutic agent for acute myelogenous leukemia. However, because of 5-azacytidine's general toxicity, other nucleoside analogs were favored as therapeutics. The finding that 5-azacytidine was incorporated into DNA and that, when present in DNA, it inhibited DNA methylation, led to widespread use of 5-azacytidine and 5-aza-2′-deoxycytidine (Decitabine) to demonstrate the correlation between loss of methylation in specific gene regions and activation of the associated genes. There is now a revived interest in the use of Decitabine as a therapeutic agent for cancers in which epigenetic silencing of critical regulatory genes has occurred. Here, the current status of our understanding of the mechanism(s) by which 5-azacytosine residues in DNA inhibit DNA methylation is reviewed with an emphasis on the interactions of these residues with bacterial and mammalian DNA (cytosine-C5) methyltransferases. The implications of these mechanistic studies for development of less toxic inhibitors of DNA methylation are discussed.
Oncogene (2002) **21,** 5483–5495. doi:10.1038/sj.onc.1205699

Keywords: DNA methylation; 5-azacytidine; DNA methyltransferase; cancer

Introduction

5-azacytidine and 5-aza-2′-deoxycytidine (ZCyd and ZdCyd – See Figure 1 for structures) were first synthesized by Piskala and Sorm (1964). ZCyd was originally developed and tested as a nucleoside antimetabolite with clinical specificity for acute myelogenous leukemia (Cihak, 1974; Sorm *et al.*, 1964). Early reports indicated that ZCyd was an inducer of chromosome breakage and a mutagen (Halle, 1968; Karon and Benedict, 1972; Landolph and Jones, 1982; Paul, 1982; Viegas-Pequignot and Dutrillaux, 1976). Because it could be activated to the nucleoside triphosphate and incorporated into both DNA and RNA, ZCyd treatment of cells led to inhibition of DNA, RNA and protein synthesis (reviewed in Vesely and Cihak, 1978). Incorporation of ZCyd into tRNA was shown to inhibit tRNA methyltransferases (Lu and Randerath, 1980), and to interfere with tRNA methylation and processing leading to defective acceptor function of transfer RNA (Lee and Karon, 1976). Since methylation also plays an important role in ribosomal RNA processing (Glazer *et al.*, 1980; Weiss and Pitot, 1974), effects of incorporation of ZCyd on RNA function and stability are likely to account for much of ZCyd's effect on protein synthesis. In addition, enzymatic deamination of ZCyd and ZdCyd yields 5-azauridine and 5-aza-2′-deoxyuridine respectively. These compounds interfere with *de novo* thymidylate synthesis, adding to cytotoxicity (Vesely *et al.*, 1969).

5-Aza-2′-deoxycytidine (ZdCyd), which is only incorporated into DNA (Li *et al.*, 1970), is at least 10-fold more cytotoxic than ZCyd for cultured cells and animals (Flatau *et al.*, 1984; Momparler *et al.*, 1984). At dose levels low enough to avoid triggering cell death, incorporation of ZCyd or ZdCyd into DNA of cultured cells leads to rapid loss of DNA (cytosine-C5) methyltransferase (DNA (C5) MTase, Dnmt) activity because the enzyme becomes irreversibly bound to ZCyt residues in DNA (Christman *et al.*, 1983; Creusot *et al.*, 1982; Taylor and Jones, 1982). Studies in bacteria, cultured cells and rodents indicate that these Dnmt-ZCyt adducts are toxic and mutagenic if not repaired (Bhagwat and Roberts, 1987; Jackson-Grusby *et al.*, 1997; Juttermann *et al.*, 1994). However, DNA (C5) MTases only bind to ZCyt or other inhibitory Cyt analogs that replace Cyt targets for methylation in their specific recognition site (Christman *et al.*, 1995; Friedman, 1986; Santi *et al.*, 1984). Since the methylation target for the mammalian maintenance methyltransferase, Dnmt1, is CpG in hemi-methylated sites (Figure 2), significant inhibition of DNA synthesis due to lack of repair of Dnmt1-ZCyt adducts does not occur for at least two cell cycles (Davidson *et al.*, 1992). In contrast, Dnmt1 becomes bound to DNA and inactivated as soon as ZCyt is incorporated into CpG sites opposite a methylated CpG site on the template strand, i.e., within a few hours of initiating treatment with ZCyd or ZdCyd. This, in turn, leads to rapid passive loss of methylation (Figure 2b) (Creusot *et al.*, 1982). In this regard, it is of interest

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to note that the level of cellular resistance to the toxic effects of ZCyd and ZdCyd is inversely correlated with the level of active Dnmt1 rather than the level of ZCyt in DNA (Flatau *et al.*, 1984; Juttermann *et al.*, 1994). The finding that cells selected for resistance to ZdCyd are still capable of incorporating ZdCyd into their DNA to the same extent as untreated cells, suggests that resistance to ZCyd toxicity occurs because the probability that Dnmt1 will encounter and become covalently bound to ZCyt in hemimethylated CpG sites is greatly reduced even when Dnmt1 levels have returned to normal (Flatau *et al.*, 1984).

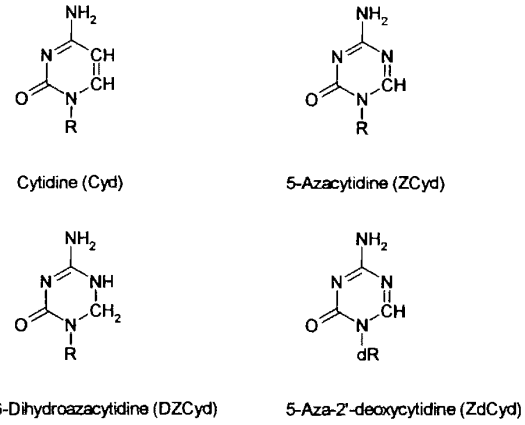

Figure 1 Structures of cytidine and its 5-aza- analogs. R = ribose. dR = deoxyribose

Role of altered DNA methylation in cancer

Reduced methylation of DNA in cancer

The first hint that there was a link between methylation and cancer came from studies showing that 5-methylcytosine (MCyt) levels were lower in DNA of tumor cells as compared to normal cells (Gama-Sosa *et al.*, 1983; Lapeyre and Becker, 1979; Lapeyre *et al.*, 1981). It was soon demonstrated that loss of DNA methylation was an early event in tumorigenesis, occurring even in preneoplastic colonic epithelium of individuals with familial polyposis coli (Feinberg *et al.*, 1988; Feinberg and Vogelstein, 1987; Goelz *et al.*, 1985). Hypomethylation of *H-ras* and *MYC* genes is common in a variety of human tumors (Fang *et al.*, 1996; Feinberg and Vogelstein, 1983; Sharrard *et al.*, 1992; Vachtenheim *et al.*, 1994), but it has not been linked to overexpression of these genes in cancers. However, severe dietary deficiency of sources of methyl groups (lipotropes: choline, methionine, vitamin B12 and folate) was found to lead to development of hepatocellular carcinoma in rats and to enhance development of carcinogen induced tumors (Ghoshal and Farber, 1984; Lombardi and Shinozuka, 1979; Mikol *et al.*, 1983; Newberne and Rogers, 1986). Although Dnmt1 activity in hepatocytes was elevated 2–3-fold, probably due to cell division induced by the methyl deficient diet, passive loss of DNA methylation occurred due to continued synthesis of DNA in hepatocytes under conditions of limiting 5-adenosyl-methionine (AdoMet) and elevated 5-adenosyl-homocysteine (AdoHcy). Decreased methylation in liver DNA could be detected within a few days of initiating the feeding of a methyl deficient diet (Christ-

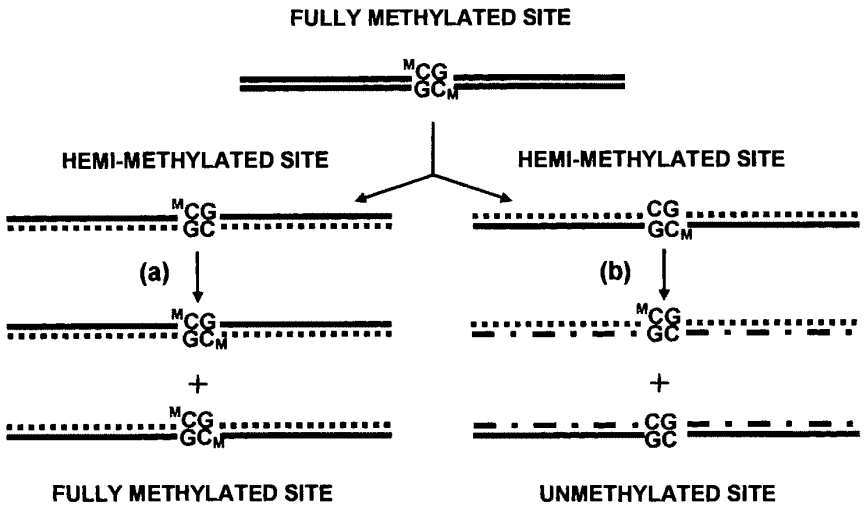

Figure 2 Fate of fully methylated sites in DNA during DNA replication. (**a**) After DNA replication, hemimethylated sites are re-methylated by the maintenance MTase, Dnmt1, restoring pre-existing patterns of methylation. (**b**) Methylation is passively lost in one of the daughter DNA molecules because Dnmt1 was not available to remethylate hemi-methylated sites created during the first round of DNA replication. This occurs in ZCyd and ZdCyd treated cells because Dnmt1 is inactivated due to covalent linkage to ZCyt residues in CpG sites in DNA

man *et al.*, 1993a,b; Dizik *et al.*, 1991; Wainfan *et al.*, 1989). Even with limited site-specific methylation analysis, it could be demonstrated that decreases in methylation only occurred in a limited subset of CpG sites. Loss of methylation at these sites persisted for at least 9 months after the animals were returned to a normal diet, suggesting that little *de novo* methylation occurred once the mitogenic stimulus of methyl deficiency was no longer present. In this rat model for preneoplastic liver and in several human tumor types, a correlation could be observed between decreased methylation levels and increased expression of specific genes (reviewed in Christman, 1995; Ehrlich, 2000).

Paradoxically, studies with cultured cells indicated that inhibitors of DNA methylation, such as L-ethionine and ZCyd, two compounds that inhibit DNA methylation by totally different mechanisms, could lead to activation of a differentiation program in a variety of human and murine cell lines derived from tumors and normal tissues (Christman *et al.*, 1977; Constantinides *et al.*, 1977; Creusot *et al.*, 1982; Mendelsohn *et al.*, 1980; Taylor and Jones, 1979; Taylor and Jones, 1982; reviewed in Zingg and Jones, 1997). In part, this apparent discrepancy was reconciled by the demonstration that in CH310T1/2 cells, where treatment with ZCyd caused the cells to differentiate into muscle, chondrocytes and adipocytes (Constantinides *et al.*, 1977), it also caused reactivation of MyoD1, a gene that plays a vital role in myocyte differentiation. Although the function of MyoD1 is not regulated by methylation during normal development, the CpG island in the regulatory region of the gene presumably became *de novo* methylated during establishment of the cells in culture (Jones *et al.*, 1990).

Increased methylation of DNA in cancer

CpG islands are stretches of DNA approximately 1 kb long that are rich in CpG and GpC dinucleotides (Bird, 1986). The CpG sites in these gene-associated regions are rarely methylated in normal cells with the exception of CpG islands of genes on the inactivated X chromosome and CpG islands associated with imprinted genes (Barlow, 1995). It is now accepted that abnormal methylation of CpG islands is not restricted to cultured cells but can also occur during aging and during tumor development (Baylin *et al.*, 1998; Issa *et al.*, 1996; Wilson and Jones, 1983). The mechanism by which hypermethylation of selected CpG islands occurs in cells undergoing an overall decrease in level of cytosine methylation remains to be resolved. However, it has now been shown that unmethylated CpG islands associated with a variety of genes become partially or fully methylated in tumors and can be reactivated by ZCyd (Table 1). Some of these genes appear to be 'bystanders' that are not expressed in either the normal tissue or the tumor arising from it but are methylated in the tumor (Silverman *et al.*, 1989). Similarly, some CpG island methylation has no effect on gene activity because it occurs in CpG islands that are not associated with the regulatory regions of genes (Jones, 1999; Nguyen *et al.*, 2001). Over the last 10 years, it has been well documented that loss of tumor suppressor gene function can occur both through mutation and through gene silencing linked to methylation of CpG island promoters (reviewed in Baylin *et al.*, 1998; Santini *et al.*, 2001). A recent examination of more than 600 primary tumor samples from 15 tumor types showed that CpG island promoters of three or more genes from a panel of 12 known tumor suppressor genes were hypermethylated in 5–10% of the samples. At least one CpG island was methylated in 80% or more of samples for each tumor type (Esteller *et al.*, 2001). Using methods that allow genome wide screening of CpG islands, it has been estimated that, on average, ~1% of CpG islands in DNA from tumor tissues are abnormally methylated (Costello *et al.*, 2000; Yan *et al.*, 2001). These studies also provide evidence for tumor specific patterns of CpG island methylation, with the percentage of CpG island methylation in individual tumors varying from 0–10% of the ~45 000 CpG islands in the human genome (Costello *et al.*, 2000). There is also evidence of silencing of the p53 tumor suppressor gene as a result of methylation of CpG sites in its 'non-CpG island' promoter and of hypermethylation of these sites in human hepatomas (Pogribny and James, 2002; Pogribny *et al.*, 2000).

There are a number of examples of treatment with ZCyd or ZdCyd leading to reactivation of function in tumor cell lines in which one copy of a gene for a tumor suppressor, a cell cycle regulator or a DNA repair enzyme is mutated and the other is normal (wild type) but inactivated by methylation. This suggests that loss of methylation induced by ZCyd or ZdCyd treatment can lead to reactivation of the same genes whose inactivation was selected for during development of a specific tumor and has stimulated interest in revisiting the use of ZdCyd and other Cyd analogs in anticancer therapy (Baylin *et al.*, 2001). This review will provide an overview of the mechanistic attributes of DNA (C5) MTases and discuss how these characteristics could explain why ZCyt in DNA affects both DNA methylation and acts to trigger apoptosis in cancer cells.

DNA cytosine (C5) methyltransferases: enzyme structure and catalytic mechanism

The catalytic mechanism of DNA (C5) MTases involves formation of a covalent bond between a cysteine (Cys) residue in the active site of the enzyme and carbon 6 (C6) of cytosine (Cyt) in DNA. This increases flow of electrons to carbon 5 (C5), with subsequent attack on the methyl group of AdoMet. Abstraction of a proton from C5 followed by β-elimination then allows reformation of the 5–6 double bond and release of the enzyme and DNA with a methylated Cyt (Figure 3a). Santi *et al.* (1984) proposed a mechanism that would account for the

Table 1 Genes with promoter CpG islands hypermethylated in tumors and reported to be reactivated with 5-azacytidine, 5,6-dihydroazacytidine or 5-aza-2′-deoxycytidine

Gene	*Reference*
14-3-Sigma	Ferguson *et al.*, 2000
AR	Izbicka *et al.*, 1999
BRCA1	Magdinier *et al.*, 2000
CD44	Sato *et al.*, 1999
CX22	Singal *et al.*, 2000
DBCCR1	Habuchi *et al.*, 1998
ECAD	Yoshiura *et al.*, 1995
ETB	Nelson *et al.*, 1997
ER-alpha	Ferguson *et al.*, 1995; van Agthoven *et al.*, 1994
FHIT	Zochbauer-Muller *et al.*, 2001
GATA	Visvader and Adams, 1993
GPC3	Murthy *et al.*, 2000
GSTP-1	Jhaveri and Morrow, 1998
H19	Barletta *et al.*, 1997; Chung *et al.*, 1996
hMLH1	Cameron *et al.*, 1999; Herman *et al.*, 1998; Plumb *et al.*, 2000
IGF2	Hu *et al.*, 1997
KAI1	Sekita *et al.*, 2001
MDGI	Huynh *et al.*, 1996
MDR1	Kantharidis *et al.*, 1997
MGMT	Mitani *et al.*, 1989
MUC2	Gratchev *et al.*, 2001
MYOD1	Chen and Jones, 1990
NM23-H1	Hartsough *et al.*, 2001
P15 (CDKN2B)	Cameron *et al.*, 1999
P16 (CDKN2)	Lee *et al.*, 1997; Merlo *et al.*, 1995; Myohanen *et al.*, 1998; Timmermann *et al.*, 1998; van der Velden *et al.*, 2001
P57KIP1	Shin *et al.*, 2000
P73	Kawano *et al.*, 1999
PAX6	Salem *et al.*, 2000
RAR-beta2	Arapshian *et al.*, 2000
RASSF1A	Astuti *et al.*, 2001; Dreijerink *et al.*, 2001
TGFBR1	Ammanamanchi and Brattain, 2001
THBS1	Li *et al.*, 1999
TIMP3	Cameron *et al.*, 1999
uPA	Xing and Rabbani, 1999

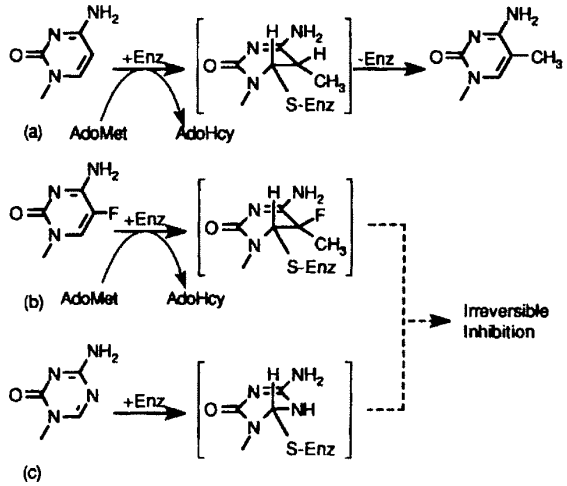

Figure 3 Formation of a 5,6-dihydropyrimidine intermediate during methylation of a target DNA containing (**a**) Cyt, (**b**) FCyt and (**c**) ZCyt

inhibitory effects of the Cyt analogs, i.e., formation of a covalent bond between the enzyme and 5-flurocytosine (FCyt) or ZCyt (Figure 3b,c) that could not be easily reversed. A number of reports provided experimental support for this mechanism of interaction between prokaryotic and mammalian DNA (C5) MTase and DNAs or oligodeoxyribonucleotides (ODNs) containing ZCyt (Christman *et al.*, 1985; Friedman, 1986; Gabbara and Bhagwat, 1995; Santi *et al.*, 1984).

It is assumed that the mammalian DNA (C5) MTases accomplish methyl transfer and are inhibited by ZCyt in the same manner as the bacterial DNA (C5) MTase, M.*Hha*I, since these enzymes share a set of conserved catalytic domain motifs (Lauster *et al.*, 1989; Posfai *et al.*, 1989). These include a prolylcystineyl active site dipeptide (region IV), a group of separate domains that come together to form the binding pocket for AdoMet (I, X) and a region (VI) containing the glutamyl residue that protonates nitrogen 3 (N3) of the target Cyt. The recognition domain that makes base-specific contacts in the major groove of DNA usually lies between motifs VIII and IX.

X-ray crystallographic analysis of a ternary complex containing AdoHcy and M.*Hha*I with the cysteine (Cys81) of the prolylcysteinyl dipeptide covalently linked to FCyt in DNA (F13) demonstrated that the

covalently linked target Cyt is flipped out of the DNA helix into the catalytic pocket of the MTase. The structure predicts that C5 of a flipped target Cyt is aligned with a bound AdoMet molecule and C6 with the thiolate residue of the active site cysteine (Cys81) in a manner that facilitates a concerted attack and methyl transfer (Klimasauskas *et al.*, 1994). As determined in modeling studies (O'Gara *et al.*, 1996a), once methyl transfer from AdoMet to C5 occurs, a tension develops between the methyl group on C5 of the flipped MCyt and adjacent amino acids Pro80 and Cys81, destabilizing the enzyme DNA complex sufficiently to allow release of the methylated product.

There is as yet no high resolution X-ray crystal structure of M.*Hha*I complexed with a ZCyt target in DNA. However, the enzyme has been crystallized in a ternary complex with AdoHcy and a synthetic oligodeoxyribonucleotide (ODN) containing a 5,6-dihydroazacytosine (DZCyt) residue as target in a hemimethylated site (DZ13 – Figure 4a; Sheikhnejad *et al.*, 1999). The only difference between DZCyt and ZCyt is the degree of saturation of the bond between C5 and C6 (Figure 1b,c). The completely saturated C5-C6 bond in DZCyt makes it an analog of the covalently bound transition state of Cyt and ZCyt formed prior to methyl transfer, while the sp^3 character of C6 abrogates nucleophilic attack. The structure of the protein and DNA components in the DZ13 complex closely approximate those of HM13, a complex of AdoHcy, M.*Hha*I with an identical ODN containing a Cyt residue as target in a hemimethylated site. While no methylation of Cyt can occur in either complex, there is a major difference between HM13 and DZ13 – the distance between C6 and the sulfur atom of Cys81. In DZ13 this distance is 3.1 Å (Figure 4a) compared to 2.6 Å in HM13 and 1.8 Å for the covalently linked C5 and Cys81 in F13 (Figure 4b). It has been noted that, even in the absence of methyl transfer in HM13, both C5 and C6 of the Cyt target take on some sp^3 character and that the reduced distance between C6 and Cys81 suggest a 'partial' covalent bond formation (O'Gara *et al.*, 1996b) while the separation of these atoms in DZC indicates a complete lack of reaction between C6 and Cys81 in DZCyt (Sheikhnejad *et al.*, 1999). The distance between Glu 119 and N3 in DZ13 is 3.3 Å compared to 2.8 Å in F13 suggesting a lack of protonation of N3 in DZ13. Other contacts between the protein and base in DZ13 are similar to those of Cyt in HM13 although there is a weaker interaction between N4 of DZCyt and Glu 119. The presence of an additional hydrogen bond between the proton on N5 and water may help to stabilize DZCyt complexes. Thus, with the exception of Cys81, the relationship of DZCyt to specific amino-acid residues in M.*Hha*I in complex DZ13 should closely approximate that of an intact ZCyt target after formation of a covalent linkage.

A model for the proposed mechanism of action of M.*Hha*I is shown in Figure 5. The enzyme associates with DNA in a sequence independent manner and scans or diffuses until it encounters its recognition site (Figure 5a). Interaction with non-specific DNA can orient the cofactor, AdoMet so that its methyl group is optimally positioned with respect to the target Cyt-C5 (O'Gara *et al.*, 1999). On encountering its specific binding/recognition site, the enzyme forms an 'open' complex comprised of 'an ensemble of flipped out conformers' (Figure $5b_{1,2}$) and finally a more compact or 'closed' complex (Figure 5c) in which Ser87 in the flexible loop of the enzyme comes together with Gln 287 in the recognition domain and locks the 'flipped' target Cyt into the catalytic pocket (Klimasauskas *et*

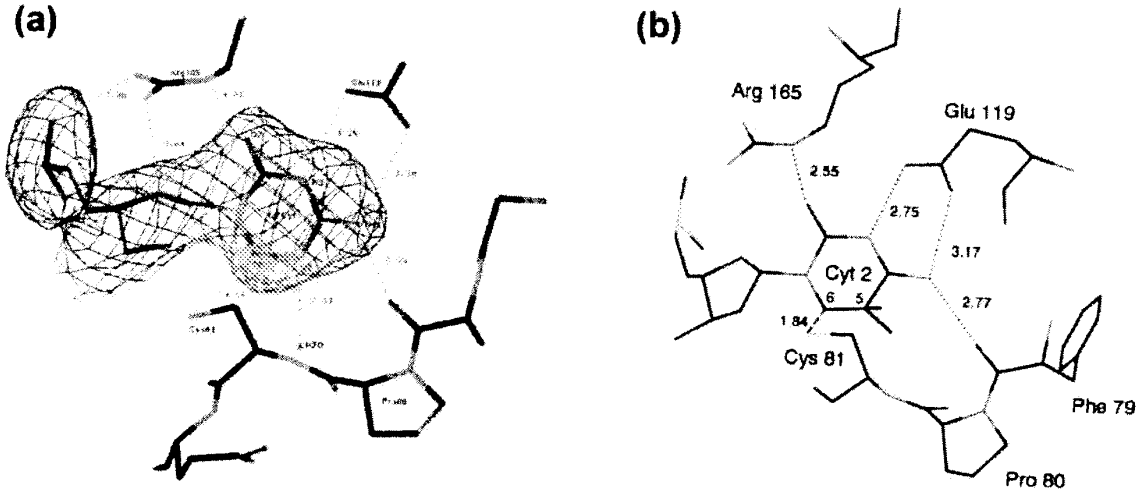

Figure 4 Comparison of the interaction of DZCyt (**a**) and Cyt (**b**) with M.*Hha*I. Difference electron density maps (F_0-$F^c\alpha_c$) superimposed on the refined coordinates with carbon atoms colored black, oxygen atoms red, nitrogen atoms blue, and sulfur atoms green, respectively. Reproduced with permission from Sheikhnejad *et al.* (1999) and Klimasauskas *et al.* (1994). These publications contain additional detail

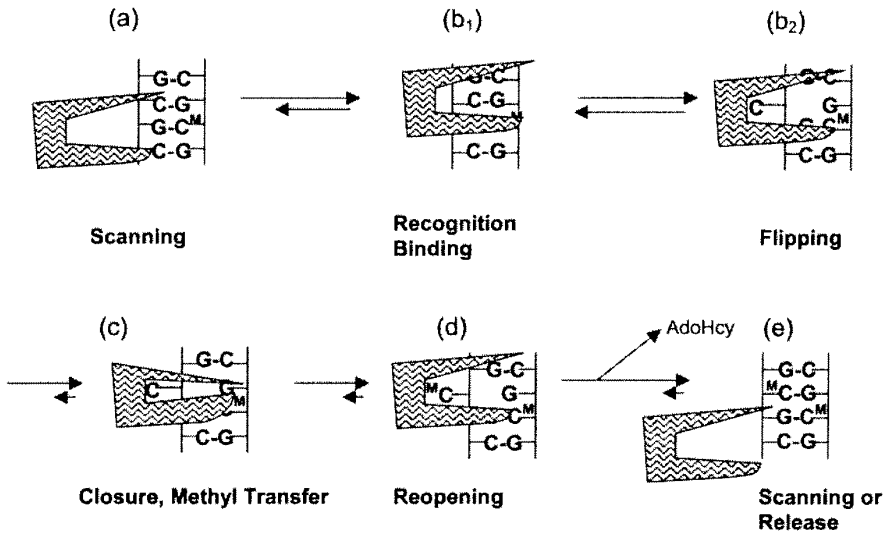

Figure 5 Model for the interactions between M.*Hha*I and DNA during the methylation reaction. It is proposed that (b_1) and (b_2) represent the two extremes for 'open' conformers of M.*Hha*I : CytODN complexes and that (c) represents the conformation of the 'closed' complex. When AdoMet is present methyl transfer to Cyt destabilizes the ternary complex leading to reopening and release of the substrate and AdoMet. In the presence of AdoHcy the equilibrium between the 'closed' complex (c) and the 'open' complex (d) is greatly shifted to the left, since no methylation occurs. Covalently linked ZCyt and DZCyt form these stable 'closed' complexes in the absence of cofactor (see text for details)

al., 1998). The 'open' and 'closed' complexes are distinguished by their electrophoretic mobility on non-denaturing gels, where the 'closed complex' migrates significantly faster than the 'open' complex (Klimasauskas and Roberts, 1995). Normally, when AdoMet is present, ternary complexes with M.*Hha*I and DNA with a Cyt target are not detected due to release of the enzyme after methylation of the Cyt residue. However, highly stable ternary complexes between M.*Hha*I and Cyt targets ($t_{\frac{1}{2}}$ of dissociation >9 h) can be formed when the enzyme lacks an active site Cys81 (Mi and Roberts, 1993), providing evidence that methylation is necessary to destabilize the closed complex with Cyt (Figure 5d). Analysis of the crystal structure of the ternary complex between M.*Hha*I and a 5MCyt target (M13) (O'Gara *et al.*, 1996a), suggest that the steric tension between the C5-methyl group and Pro80 could play a role in opening of the complex and release of the methylated product (Figure 5e).

Although it is still not understood how the flipping of the base is initiated, it is clear that M.*Hha*I does not 'recognize' the base that is to be flipped, but rather the sugar phosphate backbone and the sequences flanking the target. M.*Hha*I forms 'closed' ternary complexes with AdoHcy and DNAs containing a variety of purine and pyrimidine bases substituted for the Cyt target in the GCGC recognition site of the enzyme (Klimasauskas and Roberts, 1995; O'Gara *et al.*, 1999; Yang *et al.*, 1995). Even in the absence of a base, the sugar-phosphate backbone of DNA can assume a 'flipped-out' conformation in ternary complex with M.*Hha*I (Cheng and Roberts, 2001). However, the stability of the 'closed' complexes with bases other than Cyt varies greatly and, with few exceptions, formation of 'closed' complexes requires the presence of AdoHcy (Klimasauskas and Roberts, 1995; Sheikhnejad *et al.*, 1999; Yang *et al.*, 1995). The rank order of binding strengths and rates of dissociation, in conjunction with the data from X-ray crystallographic analysis of a large array of M.*Hha*I ternary complexes (Cheng and Roberts, 2001), suggest that suboptimal interactions between non-cytosine bases and the amino acid contacts in the active site play a critical role in reducing complex stability, favoring reopening of the complex and release of the flipped base (Sheikhnejad *et al.*, 1999).

Structural determinants of the stability of 'closed' complexes with ZCYT-ODNs

There are at least three exceptions to the rule that co-factor is necessary for formation of 'closed' complexes between M.*Hha*I and potential substrates. Electrophoretic mobility on native gels demonstrated that M.*Hha*I complexes with ODNs containing DZCyt as a replacement for the Cyt target of M.*Hha*I had the same 'closed' mobility in the presence of AdoMet or AdoHcy or in the absence of cofactor (Sheikhnejad *et al.*, 1999). Identical behavior was noted for ODNs with abasic carbocyclic and furanose sugars in place of Cyt (Wang *et al.*, 2000). An improved method for the automated synthesis of ODNs containing ZCyt (García *et al.*, 2001) allowed us to examine the formation of complexes between M.*Hha*I and ZCyt-ODN and to determine that formation of 'closed' complexes with ZCyt-ODN is also independent of cofactor (Brank *et al.*, in preparation). However, unlike complexes with DZCyt and AP-ODNs, which have monophasic

dissociation curves, the dissociation process for M.*Hha*I:ZCyt-ODN complexes was biphasic. A small proportion of complexes dissociated with a $t_{\frac{1}{2}}$ of 1–1.4 h, regardless of the presence or absence of cofactor. However, in the second phase, the $t_{\frac{1}{2}}$ for complex dissociation ranged from >4 days in the absence of cofactor to >2–3 weeks for complexes formed in the presence of AdoMet or AdoHcy.

As diagrammed in Figure 6, there are a variety of breakdown products of ZCyt that can form in aqueous solution or after covalent linkage of ZCyt to DNA MTase. Analysis by mass spectrometry of ODNs used in our studies indicated that the purified ODNs contained only two forms of ZCyt, the intact base (I) and a small amount of the ring-open form that has undergone loss of C6 as formate (IVa) (García *et al.*, 2001). Thus, it is most probable that the first phase of dissociation represents rapid dissociation of enzyme from ODNs containing IVa and the second phase, an extremely slow dissociation of ZCyt-ODN covalently linked to enzyme (IIb). Complexes formed between M.*Hha*I and ODNs containing the ring-open form IVa (ZCyt-IVa-ODN) would be expected to be much less stable than those resulting from covalent binding to intact ZCyt (IIb). While several of the structural features of the Cyt ring interaction with amino acids in the catalytic pocket of M.*Hha*I could be maintained in ZCyt-IVa-ODN, i.e. the N3,N4 interactions with Glu119, the O2 and phosphodiester backbone interactions with Arg165 and the N3 amino group interaction with Pro80 (O'Gara *et al.*, 1996a), ZCyt-IVa ODN has lost any possibility of stabilization through formation of a partial or complete covalent bond between C6 of ZCyt and Cys81 of M.*Hha*I. In addition, formation of an alternate ring structure through intramolecular hydrogen bond formation (Structure V; Jackson-Grusby *et al.*, 1997) could interfere with enzyme/DNA interactions such as the one between O2 and Arg165. The predicted poor fit of V in the catalytic pocket would suggest that it should be similar to a mismatched base in its stability under non-denaturing conditions. The $t_{\frac{1}{2}}$ for the rapid phase of dissociation of ZCyt complexes and magnitude of the enhancement of binding by AdoHcy (~50%) is similar to that of ODNs with Ade and Ura replacing Cyt (Sheikhnejad *et al.*, 1999, and unpublished AS Brank, JK Christman). In this regard, it may be significant that a small proportion of binary complexes with ZCyt-ODN migrate in native gels with the slower rate characteristic of 'open' complexes, while only 'closed' complexes are seen in the presence of cofactor (Brank *et al.*, in preparation).

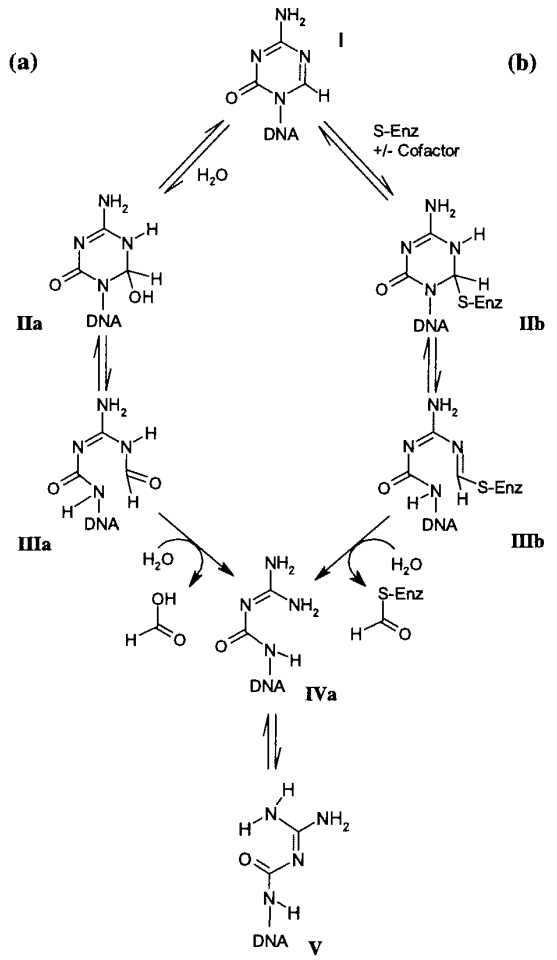

Figure 6 Reaction pathways for ring opening and hydrolysis of 5-azacytosine residues in DNA in solution (**a**) and after covalent linkage to the active site cysteine of a DNA (C5) MTase (**b**). Note that the end result of these pathways is the same, an unliganded DNA with a potentially mutagenic base residue (V). However, the interaction in path (**b**) leads to inactivation of the enzyme through formylation of the catalytic cysteine

The observation that M.*Hha*I:ZCyt-ODN complexes with an extremely slow rate of dissociation form in the absence of cofactor or the present of AdoHcy is consistent with Santi's proposal (Santi *et al.*, 1984) that addition of a proton at N5 leads to formation of a stable but slowly reversible covalent bond between DNA (C5) MTases and ZCyt in DNA and that methylation of ZCyt residues is not necessary for covalent bond formation. In agreement with this, transfer of radiolabeled methyl groups from AdoMet to ZCyt-ODNs by M.*Hha*I was barely detectable (AS Brank and JK Christman, unpublished data). A standard test of covalent linkage between FCyt residues in DNA and MTasesis to heat the complexes to 95°C in the presence of these denaturants. None of the DZCyt-, ZCyt- and AP-ODNs complexes formed with M.*Hha*I in the presence or absence of cofactor survive this treatment (Brank *et al.*, in preparation; Sheikhnejad *et al.*, 1999). This was expected because no covalent bonds can be formed with DZCyt- or furanoses, and ring opening of ZCyd is promoted by heating (Beisler, 1978). However, ODNs containing any of these three targets form 'closed' complexes that are stable in the presence of SDS and 2-mercaptoethanol at 22°C regardless of the presence or absence of

cofactor. None of the 'closed' ternary complexes of M.*Hha*I with AdoHcy and natural bases (Cyt or mismatched bases) are stable under these conditions. Even more striking, the complexes involving M.*Hha*I and ZCyt-ODNs migrate with a higher mobility in denaturing SDS gels because of a reversible change in the conformation of M.*Hha*I that results from its interaction with the ZCyt target (Figure 7). The mobility of this complex differs markedly from that of the complex resulting from M.*Hha*I methylation of and covalent linkage to FCyt-ODN. Without heating, only a small proportion of the ternary M.*Hha*I : FCyt-ODN : AdoMet complexes migrate more rapidly than free enzyme (Figure 7, lane 4). Rather, there is a 'smear' of slower moving complexes, with the slowest migrating at the rate expected for M.*Hha*I covalently linked to FCyt-ODN. Heating at 95°C converts all of the covalently linked complexes to this form (Figure 7, lane 3). In contrast, there are no slower moving complexes formed with ZCyt-ODNs (Figure 7, lanes 6,7) and, as the complex is heated, ZCyt ODN is released by a process without conversion to a detectable 'open' form (lane 5).

If it is assumed that ZCyt in both binary and ternary complexes of ZCyt-ODN with M.*Hha*I is covalently linked to the enzyme and has the same basic structure as DZCyt in DZ13, the major difference between ZCyt- and FCyt-ODNs in the complexes is evident. Formation of stable complexes between M.*Hha*I and FCyt-ODNs requires transfer of a methyl group to the FCyt target while methylation of the ZCyt target in ZCyt-ODNs is not only unnecessary but is actually a rare event. Thus, at 22°C, under denaturing conditions, the covalent complex between enzyme and methylated FCyt-ODN takes on a variety of conformations ranging from tightly closed to fully 'open' because the presence of the (C5)-methyl group destabilizes the complex. With increased temperature, the equilibrium is shifted to the fully 'open' form (Figure 7 and unpublished data, AS Brank and JK Christman). All of the M.*Hha*I : ZCyt-ODN complexes, regardless of the presence or absence of co-factor, migrate with the same increased mobility that we postulate is the result of the 'closed' configuration assumed by M.*Hha*I when the active site loop (residues 80–90) locks the flipped-out target into catalytic pocket. M.*Hha*I : DZCyt-ODN complexes have an identical mobility to M.*Hha*I : Z-Cyt-ODN complexes in the presence of SDS and 2-mercaptoethanol at 22°C (DZCyt), but are less stable to heating. This provides support for the proposal that ZCyt in the high mobility M.*Hha*I : ZCyt-ODN complex has the same non-aromatic ring structure with saturation of the 5,6 double bond between C6 and the N5 (Structure IIb) and suggests that the added stability and slower dissociation rate of the M.*Hha*I : Z-Cyt-ODN complex ($t_{\frac{1}{2}} > 100$ h) relative to the M.*Hha*I : DZCyt-ODN complex ($t_{\frac{1}{2}} < 4$ h in the presence of AdoMet) is due to the covalent bond. The slow but measurable dissociation of ZCyt-ODN from M.*Hha*I : ZCyt-ODN complexes observed in our studies could be related to the rate at which covalently linked ZCytIIb is converted to either form I or form

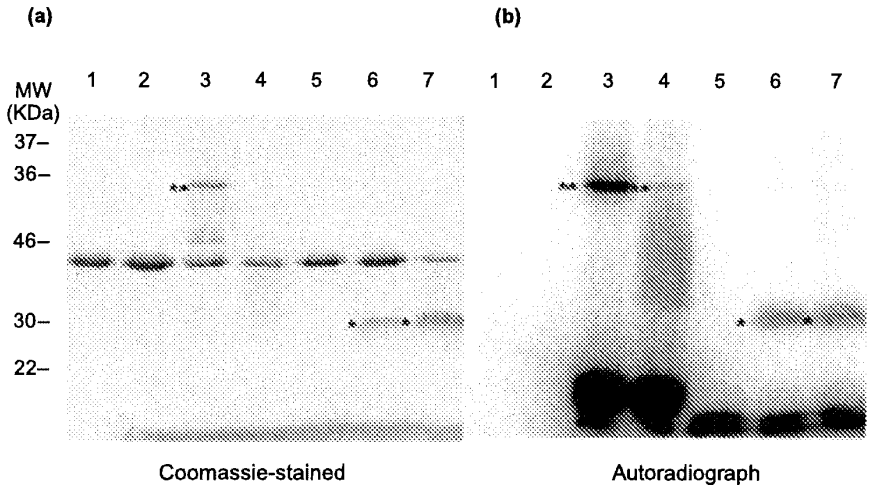

Figure 7 Comparison of the SDS–PAGE mobility of M.*Hha*I and complexes between M.*Hha*I and FCyt and ZCyt. M.*Hha*I alone (lanes 1, 2) or in mixtures containing 2.96 μM radiolabeled FCyt- (lanes 3 and 4) of ZCyt-ODN (lanes 5–7), 1.35 μM M.*Hha*I, and 200 μM AdoMet were incubated for 1 h at 37°C to allow complex formation. After addition of 1% SDS and β-mercaptoethanol, the samples were either held at room temperature (lanes 2, 4, 7) or heated for 5 min at 95°C (lanes 1, 3, 5) or 75°C (lane 6). After electrophoresis through a 10% SDS–polyacrylamide gel, M.*Hha*I protein was visualized by staining with Coomassie blue (**a**). The complexes containing bound ODN were visualized by autoradiography (**b**). The migration of molecular weight markers is indicated. *Indicates 'closed' complexes between ODN and M.*Hha*I, **indicates the completely 'open' complex. The increase in mobility (*) with binding of ZCyt-ODN is visible in both (**a**) and (**b**) as is the decrease in mobility (**) caused by methylation of FCyt-ODN. Note that FCyt-ODN is 24 bp long and ZCyt-ODN 13 bp long

IVa. However, hydrolytic conversion of covalently linked-ZCyt to form IVa is irreversible and leads both to scission of the covalent linkage to M.*Hha*I and the formation of a remnant of the base that should destabilize the 'closed' conformation (above). This route for dissociation coupled with absence of methyl transfer would account for the complete absence of M.*Hha*I : ZCyt-ODN complexes with partially or fully 'open' conformation.

It is important to note that Dnmt1 : FCyt- and ZCyt-ODN complexes exhibit similar properties to M.*Hha*I : FCyt- and M.*Hha*I : ZCyt-ODNs with regard to stability and altered mobility during electrophoresis on denaturing gels (Figure 8). This means that inferences drawn from our studies of interactions between M.*Hha*I and ZCyt in DNA are relevant to the understanding of the effects of ZdCyd or DZCyd incorporation into DNA during cancer therapy.

Implications of the interactions between DNA (C5) MTases and Cyt analogs in DNA for cancer therapy

Although there are a variety of compounds that can inhibit DNA methylation in mammalian cells (Zingg and Jones, 1997), the only DNA MTase inhibitors that have undergone extensive clinical trial are ZCyd, DZCyd (5,6-dihydroazacytidine, DHAC) and ZdCyd (Decitabine). All three compounds are inhibitors of DNA methylation only when incorporated into DNA. As noted, ZdCyt is more potent an inhibitor of DNA methylation than ZCyt because it is only incorporated into DNA. DZCyd, which was synthesized as a more stable analog of ZCyd (Beisler, 1978) is at least an order of magnitude less potent than ZCyt in blocking *in vivo* methylation (Jones and Taylor, 1980; McGregor *et al.*, 1989). This has been ascribed to limited incorporation DZCyt into DNA because it is inefficiently phosphorylated by cytidine kinase (McGregor *et al.*, 1989).

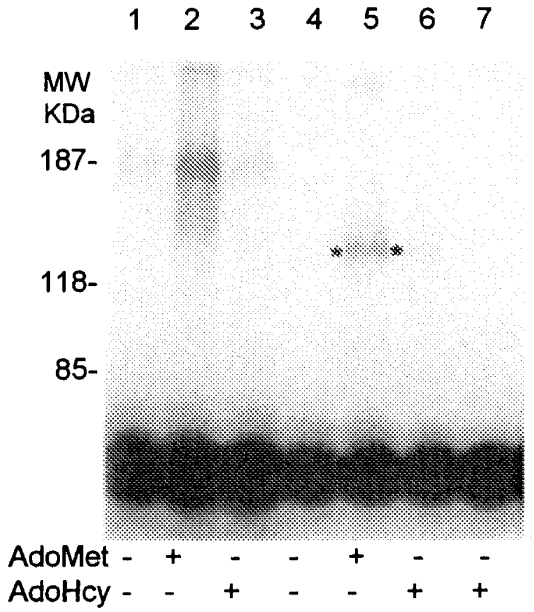

Figure 8 Comparison of the SDS–PAGE mobility of complexes between purified rDnmt1 (Brank *et al.*, 2002) and FCyt- (lanes 1–3), ZCyt- (lanes 4–6) or Cyt-ODN (lane 7). Details as in Figure 7. Samples were electrophoresed on 6% SDS-polyacrylamide gels without heating. The increase in mobility with binding of ZCyt ODN is indicated (*). Note the broad smear of conformers is detected for covalently linked methylated FCyt-ODN and that only free ODN was detected in lane 7

ZCyt has been under clinical study as a cancer treatment since the 1960s. The NCI cancer clinical trial database lists a total of 71 closed trials for ZCyd, along with four open Phase I or Phase II trials of ZCyd or ZCyd in combination with the histone deacetylase inhibitor, phenylbutyrate or a radioprotective agent, amifostine. A total of 13 trials of ZdCyd are listed, with three still open and a total of 16 for DHAC, all closed. The range of diseases treated with ZCyd includes β-thalassaemia, sickle cell anemia, leukemias (CML, AML, myelodysplasia), metastatic lung cancer, EBV-associated malignancies, androgen insensitive prostate cancer, metastatic lung cancer, cervical cancer, testicular cancer, colorectal, head and neck, renal, malignant melanoma, ovarian cancer and AIDs. However, the greatest successes have come with treatment of advanced myelodysplastic syndrome, CML and AML (reviewed in Santini *et al.*, 2001). The number of clinical trials of anti-tumor effects of ZCyd, ZdCyd and DHAC on solid tumors is relatively small and reported responses are generally low and/or lower than conventional therapy. Nevertheless, a recent pilot Phase I/II trial of ZdCyd for metastatic lung cancer indicated 'some clinical activity' (Momparler *et al.*, 1997).

Fifteen years ago, Glover *et al.* (1987) summarized a review of ZCyd trials with the comment 'Azacitidine has been undergoing clinical trials for almost 20 years and is internationally considered to have a useful place in the treatment of acute nonlymphocytic leukemia. However, its role in the various combinations for induction, intensification, maintenance, or relapse regimens has not yet been clearly defined'. It is still difficult to make the determination as to whether ZCyd or Decitabine act primarily through cytotoxicity or through effects on DNA methylation (Daskalakis *et al.*, 1999). With high levels of incorporation of ZCyt into DNA, it is likely that cytotoxicity predominates due to formation of Dnmt : ZCyt adducts in DNA. Clonal outgrowth of cells resistant to ZCyd or Decitabine toxicity, either because they are not synthesizing DNA during the period of treatment or because they have few methylated CpG targets, could easily account, not only for observed switches to a 'normalized' karyotype, but also for differences in methylation patterns pre- and post-treatment. It is of interest in this regard that a recent report suggests that Dnmt1 : ZCyt adducts in DNA can activate the p53 DNA damage response pathway in cells with wild-type p53 and that p53/p21

activation could contribute to clinical efficacy of ZCyt and ZdCyd in cancers such as CML where functional p53 is often retained (Karpf *et al.*, 2001).

Our studies with ODNs containing ZCyt and DZCyt suggest that these inhibitors will have different effects on DNA repair and mutation. In mammalian cells, covalent ZCyt complexes are toxic (Juttermann *et al.*, 1994) and after resolution by hydrolysis leave a base remnant that is mutagenic (Jackson-Grusby *et al.*, 1997). DZCyt is stable in DNA but cannot form covalent complexes with DNA MTases. Further although it forms 'closed' complexes with M.*Hha*I, AdoMet has a much weaker capacity for stabilizing these complexes than does AdoHcy (Sheikhnejad *et al.*, 1999). Under physiological conditions, AdoMet is generally well in excess of AdoHcy (Shivapurkar and Poirier, 1983). Thus, reversibility of Dnmt1 : DZCyt-DNA complex formation and the low efficiency of incorporation of DZCyd into DNA may be equal contributors to the low potency of DZCyd as an *in vivo* inhibitor of methylation. However, the reversibility of DZCyt complex formation should also allow normal DNA replication with incorporation of G opposite the analog, suggesting that treatment with DZCyt will have a much lower potential for inducing mutations than treatment with ZCyt.

Perspectives and future directions

Considering that both ZCyd and ZdCyd have the common side effect of inducing nausea, vomiting, diarrhea and myelosuppression that limit doses and duration of treatment and that both have the potential to form mutagenic lesions, it is clear that better inhibitors of DNA methylation are needed for clinical use. While DZCyd has the advantage of lower toxicity and mutagenic potential, it has not proven to be particularly effective at inducing a therapeutic response. With a better understanding of how mechanism based inhibitors interact with DNA methyltransferases, it should be possible to design small 'decoy' DNAs containing these inhibitors for use as therapeutics (Brank *et al.*, in preparation; García *et al.*, 2001; Sheikhnejad *et al.*, 1999; Wang *et al.*, 2000).

Dnmt1, the most abundant of the three catalytically active DNA MTase in mammalian cells (Figure 9) and the best studied, is assumed to be the major target for inhibition by Cyt analogs in DNA. As summarized in Figure 9 and reviewed in Robertson (2001) Dnmt1 interacts with a number of proteins that link the enzyme to the nuclear matrix, target it to replication foci and repair sites. In addition, there are sites for binding of histone deacetylases and other proteins involved in transcriptional repression. The finding that ZCyt can induce the same kind of major conformational change in Dnmt1 as it does in M.*Hha*I suggests an alternate mechanism for the effect of ZCyt and DZCyt in DNA on gene expression, i.e., altering Dnmt's interaction with other proteins. For example, it will be important to determine whether the 'closed'

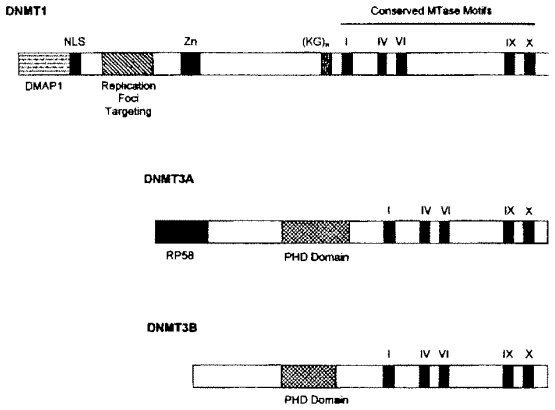

Figure 9 Structure of the catalytically active mammalian DNA methyltransferases. All three enzymes have a regulatory and catalytic domain. The conserved motifs of the catalytic domain (Roman numerals) are discussed in the text. Other structural features of Dnmt1 are the domains for nuclear localization, targeting to replication foci, and zinc binding. Binding regions for proteins involved in linkage to the nuclear matrix (p23 (Zhang and Verdine, 1996), annexin V (Oshawa *et al.*, 1996)) or transcriptional repression (HDAC1 (Fuks *et al.*, 2000), HDAC2-DMAP1 (Rountree *et al.*, 2000), pRb/DMAP1, (Robertson and Wolffe, 2000)) and targeting to replication and repair sites (PCNA (Chuang *et al.*, 1997)) are indicated. Dnmt3A and 3B share a cysteine rich PHD (plant homeodomain) region (Aasland *et al.*, 1995) and regulatory regions binding with transcriptional repressors interacting with HDAC1 (Bachman *et al.*, 2001; Fuks *et al.*, 2001)

conformation induced in Dnmt1 and other DNA MTases when bound 'decoy' ODNs containing ZCyt and DZCyt in CpG sites weakens interactions between Dnmt1 and transcriptional repressors. In the cell, this could result in both inhibition of methylation and dissociation of HDAC's, leading to rapid remodeling of chromatin and resumption of gene expression. Similarly, weakened interactions with proteins at the replication foci or in the nuclear matrix could profoundly affect maintenance of methylation required for continued compaction of inactive chromatin and nuclear localization of Dnmt1.

Nothing is known about the effect of ZCyt and DZCyt in DNA on the activity or conformation of Dnmt3a and 3b. These recently identified *de novo* methyltransferases would be predicted to have the same response to these inhibitors as M.*Hha*I and Dnmt1 but this remains to be proven. Theoretically, Dnmt3a and 3b could be much more sensitive to the inhibitory effects of ZCyt and DZCyt incorporated into DNA since they are randomly incorporated in place of Cyt and, unlike Dnmt1, Dnmt3a and 3b are capable of methylating Cyt residues that are not in CpG sites (Aoki *et al.*, 2001; Gowher and Jeltsch, 2001). However, this difference in site specificity should be an advantage in development of specific inhibitors of *de novo* methylation that could potentially prevent remethylation of genes activated by use of Dnmt1 inhibitors. The better our understanding of the similarities and difference in the mechanisms by which

inhibitors of DNA methylation affect the catalytic function of Dnmt1, 3a and 3b and protein interactions of these Dnmt's and the inactive Dnmt-like proteins, the greater the likelihood of developing novel anti-cancer drugs that can reactivate genes silenced in tumor cells.

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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) [1,2,3]

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SUMMARY

Fifty-eight patients with metastatic cancer of the breast were randomly allocated to receive 130 mg/m² of CCNU orally every 6 weeks or 60 mg/m² of 5-azacytidine intravenously daily for 10 days and twice weekly thereafter. Patients who failed to respond were treated on one of two dose schedules of cyclophosphamide. CCNU was well tolerated with the major toxic effect being delayed hematologic suppression. 5-Azacytidine produced intense gastrointestinal toxicity that often prevented maintenance therapy. There were few responses to either drug and only two patients responded to treatment with cyclophosphamide. Immunologic skin testing did not correlate with the extent of disease or the length of survival but the few responders had strong delayed hypersensitivity reactions.

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[3] 5-Azacytidine: CAS reg. No. 320-67-2; *s*-triazin-2-(1*H*)-one, 4-amino-1-β-D-ribofuranosyl-.

CCNU: CAS reg. No. 13010-47-4; urea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-.

Cyclophosphamide: CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate.

Drugs used in this study were supplied by Cancer Therapy Evaluation, DCT, NCI.

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Chemotherapeutic palliation is needed at some time in almost all patients with metastatic cancer of the breast (1). 5-Fluorouracil (5-FU) [9] and cyclophosphamide have been the most effective single agents, inducing remissions in approximately 25% of the patients (2, 3).

This cooperative study between the Albany Medical College (AMC) and the Roswell Park Memorial Institute (RPMI) was undertaken as a part of the plan of the DCT, NCI, in an effort to seek more effective single agents for the treatment of breast cancer.

5-Azacytidine and CCNU were the drugs chosen for trial. Weiss et al (4), using 5-azacytidine in a schedule similar to the one employed in this study, had noted seven of 11 objective responses and minimal toxicity in patients with breast cancer limited to soft tissues. CCNU had been shown to be effective in lymphomas (5) and glioblastomas (6) with

[9] 5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8.

some usefulness in gastrointestinal tumors (7) but had not received adequate trial in breast cancer. Patients whose disease progressed after having received one of these drugs were eligible for treatment with the "standard" agent, cyclophosphamide.

METHOD

Patients with measurable metastatic breast cancer no longer amenable to standard surgical and radiotherapeutic techniques who had not previously received these drugs were admitted to the study after submitting informed written consent. Patients were evaluated by history, physical examination, serum chemical profile, and chest and skeletal radiographs. Patients were stratified on the basis of predominant site of disease, tumor-free interval, and menopausal status (table 1) and were randomized to their treatment schedule by the closed-envelope technique.

The majority of evaluated patients were more than 1 year postmenopausal and had had a tumor-free interval, after initial treatment, of over 1 year (table 1). Patients with dominant osseous and visceral disease were equally represented and those with only soft tissue disease made up 22% of the entered patients. Six patients on each schedule had received prior cytotoxic chemotherapy with a single drug other than those used in this study. Eight patients from AMC and 18 from RPMI had had prior adrenalectomies and 28% of these patients had responded to the procedure.

The primary treatment phase compared the effectiveness of 5-azacytidine with that of CCNU. When disease progression occurred the patients were treated with one of two dose schedules of cyclophosphamide. 5-Azacytidine was given at a dose level of 60 mg/m² (not to exceed 100 mg) as an intravenous (iv) bolus each day for 10 days. A maintenance dose of 100 mg/m² (not to exceed 150 mg) was given twice weekly starting after the amelioration of hematologic toxicity from the initial course. CCNU was given at a dose level of 130 mg/m² orally and the dose was repeated every 6 weeks if the white blood cell count (wbc) was over 4000 cells/mm³ and the platelet count was over 75,000 platelets/mm³. The dose was delayed 2 weeks for patients with counts below these levels.

In the secondary treatment phase cyclophosphamide was given in one of two schedules: schedule A was 1 g/m² iv repeated every 3 weeks, and schedule B was 400 mg/m² iv daily for 4 days followed by a maintenance dose of 50 mg/day given orally. The dose was halved if the wbc fell between 5000 and 3000 cells/mm³ and was interrupted if the wbc fell below 3000 cells/mm³.

A complete remission was defined as a disappearance of all measurable disease. A partial remission was indicated if there was at least a 50% reduction in the sum of the product of the perpendicular diameters of all measured lesions in the absence of progressive disease. Progressive disease was defined as the occurrence of new disease or an increase in measured lesions to at least 125% of their original size. A treatment of at least 8 weeks was required before determining that the tumor was unresponsive.

The majority of patients were evaluated for response to skin testing before treatment. At

TABLE 1.—Stratification of patients with measurable metastatic cancer of the breast

Menstrual category	Total No. of patients	Dominant disease *					
		Visceral		Osseous		Soft tissue	
		<1 yr TF	>1 yr TF	<1 yr TF	>1 yr TF	<1 yr TF	>1 yr TF
<1 Yr postmenopausal	11	2	3	1	2	1	2
>1 Yr postmenopausal	43	8	8	6	12	2	7
Totals		10	11	7	14	3	9
	54	21		21		12	

*TF = the tumor-free interval from time of primary diagnosis until first recurrence.

AMC, 24 patients had a 2000-μg sensitizing dose and a 100-μg challenge dose of 2,4-dinitrochlorobenzene (DNCB) [10] which were simultaneously placed on the arm. The reaction to the challenge dose was interpreted at 14 days. If no reaction occurred, a repeat challenge dose of 100 μg was placed at the same site and read at 48 hours. A "mild" reaction was recorded if erythema or minimal induration was present in the area of the challenge application and a "strong" reaction was recorded if marked induration or blistering was noted. Twenty-four patients from RPMI were skin tested with the biologic agents intermediate purified protein derivative, Candida albicans (1:100 dilution), mumps antigen, and Varidase (10 units of streptokinase). The diameter of the induration at each site at 48 hours was scored as follows: 0 = 0–4 mm, 1 = 5–14 mm, 2 = 15–24 mm, 3 = 25–34 mm, 4 = 35 + mm. A mathematical sum of each skin test was used as the skin test score for each patient.

RESULTS

Fifty-eight patients were treated, 28 from AMC and 30 from RPMI. Fifty-four patients were evaluable for tumor response. In four patients the initial 10-day course of 5-azacytidine was interrupted because of severe gastrointestinal toxicity which prevented an adequate drug trial for antitumor effect. Equal numbers of patients were entered on each drug schedule in the primary treatment phase. Nineteen patients subsequently received cyclophosphamide on schedule A and 16 on schedule B.

Toxicity

Severe nausea and vomiting occurred in most of the patients who received 5-azacytidine, and in four patients it was so intolerable that the initial course of drug was not completed. Transient nausea and vomiting occurred in many patients shortly after taking the CCNU capsules. Hematologic toxicity was frequent but of moderate severity in patients treated with 5-azacytidine, whereas thrombocytopenia and leukopenia frequently delayed subsequent courses of CCNU (table 2). There were no drug-associated deaths. One patient treated with 5-azacytidine died 10 days after the initial course of therapy. A postmortem examination revealed myocardial and perivascular edema but showed no necrosis or fibrosis. The cause of death was probably due to hepatic failure from metastasis. Generalized weakness developed in one patient treated with CCNU and intellectual function deteriorated. No metastasis or other cause could be determined by brain scan, electroencephalography, or physical examination. Progression of disease during treatment was the cause of death in 12 patients who received 5-azacytidine and in nine patients who received CCNU. Hematologic toxicity was of moderate intensity in both regimens of cyclophosphamide but occurred more frequently in the high-dose intermittent schedule.

Response

Two of 27 patients who received 5-azacytidine

[10] 2,4-Dinitrochlorobenzene: NSC-6292; CAS reg. No. 97-00-7; benzene, 1-chloro-2,4-dinitro-.

TABLE 2.—Toxicity

Toxic effects	No. of patients receiving—* 5-Azacytidine (31)	CCNU (27)	Cyclophosphamide† A (19)	Cyclophosphamide† B (16)
Wbc <3000 cells/mm³	14	14	9	3
Platelets <100,000 platelets/mm³	3	17	2	3
Gastrointestinal toxic effects	28	19	13	6
Alopecia	1	—	13	8

*The total No. of patients treated with each drug is in parentheses.
†A = 1 g/m² q3wks. B = 400 mg/m² given iv for 4 days followed by oral therapy.

and three of 27 who received CCNU had partial remissions. There were no complete remissions. The mean duration of response was 5.5 months for both groups. Only one of the 14 patients with disease limited to soft tissue had a remission. None of the 12 patients who had received prior chemotherapy responded. The mean length of survival was 11.8 months for responders and 6.1 months for nonresponders. Four patients receiving 5-azacytidine and six receiving CCNU had stable disease for variable periods but progression eventually occurred.

A partial remission occurred in two patients treated with cyclophosphamide on schedule B. Three patients on each cyclophosphamide schedule had stable disease and the remaining patients had disease progression.

Skin Testing

Seven of the 24 tested patients entered from AMC had positive DNCB skin tests. The only strong reactions of the seven positive skin tests occurred in three of eight patients who had soft tissue disease only. The remaining four positive reactions were very mild and occurred both in patients with visceral and osseous dominant disease. Only one of four tested patients from RPMI with disease limited to soft tissue had a high skin test score. Five of the 12 patients who received prior chemotherapy had positive DNCB skin tests. The only responder at AMC had a strongly positive DNCB reaction and all three of the responders from RPMI that were skin tested had high skin test scores. The length of survival was not significantly different in patients who responded differently to DNCB or biologic skin testing.

DISCUSSION

The study design allowed some comparison to be made between the effectiveness and toxicity of the two primary agents tested in patients with advanced breast cancer as well as allowing a determination of the effectiveness of the two schedules of cyclophosphamide given as secondary therapy. In this fashion, too, patients were not denied treatment with a "standard" agent.

We were unable to confirm the optimistic report of Weiss et al (4) on the effectiveness of this dose schedule of 5-azacytidine in the treatment of breast cancer. The difference in response rate cannot readily be explained by differences in patient populations since more patients with disease limited to soft tissues were treated in the present study and fewer patients had received prior cytotoxic therapy than those reported by Weiss. Gastrointestinal toxicity from this drug was so severe that the majority of patients had difficulty completing their initial course. This characteristic of 5-azacytidine, combined with its low index of activity, severely limits its potential usefulness in the management of breast cancer.

CCNU was also relatively ineffective in our hands although it was tolerated much better than 5-azacytidine. Activity has been demonstrated in some other tumors (5–7) and Hoogstraten et al (8) reported seven of 36 partial responses in patients with breast cancer. The hematologic toxicity of CCNU on this dose schedule was of moderate intensity. Neurologic toxicity from CCNU was suggested in one patient but the pathogenesis was not determined.

Cyclophosphamide, used as a secondary treatment drug, was also ineffective when compared to the expected 28% remission rate when this drug is given as the first chemotherapy to similar patients (3). The patients in this study entering the cyclophosphamide phase had a poor clinical performance status after previous chemotherapy. Our low rate of remission may have resulted from exposure to ineffective agents which rendered the cancer unresponsive to a known effective agent. Talley et al (9) have shown that 38% of the patients who responded to treatment with either 5-FU or cyclophosphamide had another response when they were treated with the alternate drug, whereas few patients responded to the crossover drug after the initial failure to respond. A similar effect may follow previous chemotherapy with 5-azacytidine and CCNU.

Evaluation of the skin test data did not produce correlations with differences in disease extent or length of survival except that only those patients with disease limited to soft tissue had strong reactions to DNCB. The therapeutic response rate was too small to correlate defi-

nitely with skin testing, yet all of the responders had strong skin test reactions. Further application of skin test evaluation is currently in progress.

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A Phase I and Pharmacokinetic Study of Dihydro-5-azacytidine (NSC 264880)

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ABSTRACT

5,6-Dihydro-5-azacytidine (DHAC; NSC 264880) is an analogue of 5-azacytidine that does not possess the hydrolytically unstable 5,6-imino bond of the parent compound. Thus, unlike 5-azacytidine, DHAC is stable in aqueous solution and may be administered by prolonged i.v. infusion, potentially avoiding acute toxicities associated with bolus administration of 5-azacytidine. In this study, patients with advanced cancer were treated with DHAC administered as a 24-h constant i.v. infusion every 28 days. Treatment began at a dose of 1 g/sq m and was escalated to the maximum-tolerated dose of 7 g/sq m, where the limiting toxicity was pleuritic chest pain. Other toxicities included nausea and vomiting, which were not limiting. There was no evidence for myelosuppression, nephrotoxicity, or hepatotoxicity. DHAC was measured in plasma, urine, and ascites by a sensitive and specific reverse-phase high-performance liquid chromatography assay capable of detecting 50 ng of drug per ml. Steady-state plasma levels were achieved with 8 h and ranged from 10.0 to 20.5 μg of DHAC per ml at the maximum-tolerated dose. Total-body clearance of 311 ± 76 ml/min/sq m and postinfusion half-lives between 1 and 2 h were observed. Between 8 and 20% of the administered dose was excreted unchanged in urine. While ascites DHAC levels in a patient with ovarian cancer were comparable to plasma levels, postinfusion elimination was slower from this compartment than from plasma. No correlation was observed between DHAC plasma levels and duration or intensity of dose-limiting pleuritic chest pain. One patient with progressive Hodgkin's lymphoma demonstrated stabilization of disease for seven treatment cycles, and two patients with aggressive lymphoma demonstrated dramatic, although transient, disease responses. A dose of 7 g/sq m is recommended for Phase II trials of DHAC using this schedule.

INTRODUCTION

The pyrimidine nucleoside analogue 5-AC[2] (NSC 102816) was originally synthesized by Piskala and Sorm (1) as a potential inhibitor of nucleic acid biosynthesis. The drug is a ring analogue of cytidine and has been shown to interfere with both RNA and DNA synthesis (2). In clinical trials, 5-AC has demonstrated consistent antileukemic activity, inducing complete remissions in a significant number of heavily pretreated patients with acute myelogenous leukemia (3, 4). The dose-limiting toxicity of 5-AC is leukopenia, with less prominent thrombocytopenia. In addition, i.v. bolus treatment with 5-AC may induce severe gastrointestinal symptoms, fever, and occasional life-threatening hypotension (3, 5). These acute toxicities can be ameliorated or eliminated by administering the drug slowly via constant i.v. infusion (6, 7). However, 5-AC is unstable in aqueous solution, making strict dosage control in infusional therapy cumbersome (8–10).

To circumvent the problem of aqueous instability, Beisler and coworkers reduced the hydrolytically susceptible 5,6-imino double bond of 5-AC to produce DHAC (11) (Chart 1). This compound demonstrates excellent aqueous solubility and is stable in solution over a broad pH range.

In preclinical antitumor screening studies, DHAC demonstrated reproducible activity against murine L1210 and P388 leukemia. Screening in solid tumor models has confirmed activity against the human MX-1 mammary xenograft, the murine CD8F mammary tumor, and the s.c. implanted colon 38 tumor (12).

The mechanism of action of DHAC is presumed to be similar to 5-AC (13). Both drugs are incorporated into nuclear RNA, inhibit the methylation of ribosomal and transfer RNA, and inhibit the transcription of ribosomal and nuclear RNA (14). The overall effect is a decrease in the synthesis of methylated bases into RNA and impaired protein synthesis. Similar findings using both DHAC and 5-AC have been reported in Friend leukemia and Chinese hamster ovary cells and confirm drug-induced inhibition of RNA and protein synthesis as well as DNA synthesis (15). Both drugs are cell cycle specific, with S-phase cells most sensitive and stationary cells relatively drug resistant.

We report here the results of a Phase I trial of DHAC, including pharmacokinetic data.

MATERIALS AND METHODS

Patient Selection. Patient characteristics are shown in Table 1. Thirteen patients, 5 men and 8 women ranging in age from 27 to 69 years, were entered into study. All patients had pathological confirmation of cancer and had received prior chemotherapy. Prior to beginning treatment with DHAC, each patient underwent a comprehensive evaluation including complete history and physical examination and evaluation of measurable disease by the appropriate modality (physical examination, X-ray, or scan). Pretreatment evaluation also included complete blood count, with WBC differential, serum chemistries, and creatinine clearance. Complete blood counts were followed weekly while the patient was on study, and other parameters were repeated on Day 1 of each cycle. All patients had adequate pretreatment renal function as defined by a creatinine clearance of 60 ml/min or greater and serum creatinine less than 1.8 mg/dl, normal hemogram with WBC >3000/cu mm, platelet count >100,000/cu mm, normal hepatic function with bilirubin <2 mg/dl, and serum glutamicoxaloacetic transaminase <100 units/dl. All patients gave written, informed consent prior to therapy.

Drug Formulation and Dosage. DHAC was supplied as its hydrochloride salt by the Investigational Drug Branch, National Cancer Institute, Bethesda, MD. The drug was provided in 20-ml vials containing 500 mg of lyophilized DHAC powder with 300 mg of mannitol. When reconstituted

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[2] The abbreviations used are: 5-AC, 5-azacytidine; DHAC, 5,6-dihydro-5-azacytidine; HPLC, high-performance liquid chromatography; C_p, concentration in plasma; C_{ss}, plasma steady-state concentration; MTD, maximum-tolerated dose; THU, tetrahydrouridine.

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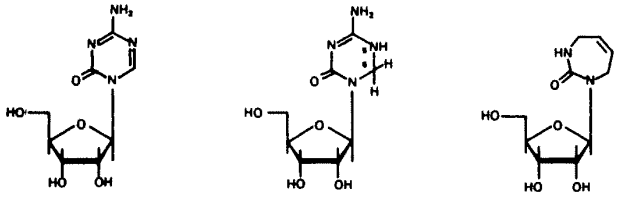

5-AC DHAC NSC 322096

Chart 1. Structures of 5-AC, DHAC, and the diazepinone riboside cytidine deaminase inhibitor NSC 322096.

Table 1

Patient characteristics

Characteristics	Measurement
No. of patients in study (eligible/evaluable)	13
Men	5
Women	8
Mean age	51 (27–69)[a]
Mean Karnofsky performance status	80 (60–100)
Tumor types	
Breast	3
Hodgkin's	3
Diffuse lymphoma	2
Melanoma	1
Ovarian	1
Colon	1
Small cell lung cancer	1
Adenocarcinoma, unknown primary	1

[a] Numbers in parentheses, range.

with 9.6 ml of sterile water for injection, United States Pharmacopeia, each ml contained 50 mg of DHAC and 30 mg of mannitol. The prescribed dose was further diluted in 500 ml of 5% dextrose and water for administration by constant infusion. When reconstituted as directed, the solution of DHAC exhibited little or no decomposition under these conditions for at least 24 h at room temperature (12).

The starting dose of DHAC was 1000 mg/sq m, administered by constant infusion over 24 h via an external pump (I-Med Model 927 infusion pump; I-Med Corp., San Diego, CA). Dose escalation was performed using a modified Fibonacci search scheme, with 3 patients entered at each dose level before escalation was performed. No patients were entered at 3.3 N, as this dosage had been safely tested in other ongoing clinical studies. No dose escalation was performed in individual patients. Treatment was continued for at least 2 cycles unless contraindicated by progressive disease or unacceptable toxicity.

***In Vitro* Stability.** Blood was obtained from a normal human volunteer by venipuncture and centrifuged for 15 min at 400 × *g* in a Dynac tabletop centrifuge (Clay Adams; Becton Dickinson Co., Parsippany, NJ). The plasma was decanted and used immediately. The appropriate volume of 1.24×10^{-2} M (3.5 mg/ml) DHAC was added to aliquots of plasma so that the final concentration was either 4.5 or 10 μg/ml. The cytidine deaminase inhibitors THU (16) and 1-β-D-ribofuranosyl-1,3,4,7-tetrahydro-1,3-diazepin-2-one (17) (NSC 322096; Chart 1) were then added to selected plasma aliquots such that the final concentration of THU was 1×10^{-4} M, and that of the diazepinone riboside was 2×10^{-5} M. A 0.5-ml plasma sample was taken from each aliquot before incubation ($t = 0$), and the remainder of the aliquot was incubated at 37 ± 0.2°C in a Dubnoff shaking metabolic incubator (Precision Scientific, Chicago, IL). For plasma with no inhibitor, 0.5-ml samples were taken at 0.25, 0.5, 1, 2, 4, and 6 h, while plasma containing THU or the diazepinone riboside was sampled at 2, 4, and 6 h. These samples were immediately frozen in dry ice and stored at −20°C until analysis.

Pharmacological Studies. Fifty μl of 4×10^{-3} M diazepinone riboside cytidine deaminase inhibitor (NSC 322096) in 0.01 M, pH 7.1, phosphate buffer were added to 10 ml of heparinized glass blood collection tubes without breaking the vacuum. These tubes were used to collect venous blood prior to the initiation of drug infusion and at predetermined times during and after completion of treatment. Blood samples were shaken thoroughly to mix inhibitor and were iced until centrifugation for 10 min at 2000 × *g* in a Sorvall RC2 centrifuge (DuPont Instruments, Wilmington, DE). The plasma was then decanted, frozen in dry ice, and stored at −20°C until analyses. Spontaneously voided urine was collected throughout the infusion period and for up to 24 h following the end of infusion. Urine volume was measured, and a 100-ml aliquot (or the entire amount if the volume was less) was frozen in dry ice and likewise stored at −20°C until analysis. Ascites was sampled in one patient via a Tenckhoff catheter immediately before treatment, at the end of the infusion, and 4 h postinfusion. These samples were treated in the same manner as plasma.

DHAC concentrations in plasma, urine, and ascites were measured by a modification of a previously reported HPLC assay (18). Urine was diluted 100× or 10× depending on whether the sample was obtained during treatment or after the end of the infusion. To a 0.5-ml aliquot of each sample were added 20 μl of 6.23×10^{-4} M (3.3 μg) 5′-chloro-5′-deoxy-5,6-dihydro-5-azacytidine as an internal standard. The sample was then diluted with an equal volume of distilled water and ultrafiltered by centrifugation at 1000 × *g* for 45 min in a Centrifree micropartition system (Amicon Corp., Danvers, MA). One-half ml of the resulting ultrafiltrate was diluted with 5.0 ml of water, and this solution was added to an 8- x 30-mm glass column of SP-Sephadex C-25 cation exchange resin (Pharmacia Fine Chemicals, Piscataway, NJ) in the NH_4^+ form. The column was then washed with 20 ml of water and eluted with 15 ml of NH_4OH, pH 9.25. The NH_4OH eluate was filtered through a 0.45-μm Millex-HA disposable filter unit (Millipore Corp., Bedford, MA), concentrated to approximately 2-ml volume, and transferred to a 10-ml round-bottomed flask. This solution was evaporated to dryness, a Teflon-coated 8-mm stirring bar was added to the flask, and the residue was dried *in vacuo* for 10 min. A hot air blower was used to warm the flask for the last 2 min to ensure complete drying. Chromogenic derivatization and HPLC analysis were carried out as described previously (18).

Standard curves for DHAC in plasma or urine were prepared for each patient's samples by addition of known amounts of DHAC to the corresponding pretreatment plasma or 10X-diluted pretreatment urine. These spiked standards were processed in the same manner as above. Appropriate standard curves were constructed for each day's analysis and typically covered the range 0 to 20 μg/ml for plasma and 0 to 50 μg/ml for urine. These curves were the best straight lines defined by least-squares regression analysis and possessed correlation coefficients greater than 0.998. The limit of quantitation ($S/N = 5$) for this modified procedure using only one column chromatography step was 0.5 μg/ml, although smaller amounts of DHAC could be detected. The plasma standard curve was also used for the ascites samples. For the few samples that were below the limit of quantitation of the modified method, the previously described 2-column workup procedure was applied (18). The limit of quantitation for this more time-consuming assay was 50 ng/ml.

Kinetic Calculations. C_{ss} was the average of measured DHAC plasma levels once these concentrations reached a plateau at about 8 h into the infusion. The apparent total-body clearance was then defined as equal to the rate of infusion divided by C_{ss}. The postinfusion C_p *versus* time data were then fit to the biexponential function representing a 2-compartment open model by an iterative nonlinear least-squares regression through the MLAB computer program (19).

RESULTS

Toxicity. Thirteen patients were treated with DHAC during this study for a total of 26 cycles of therapy. All patients were evaluable for toxicity; there were no drug-related deaths.

Dose-limiting toxicity consisted of severe pleuritic chest pain which first emerged at a dose of 5000 mg/sq m and became dose limiting at 7000 mg/sq m. Severe chest pain was reported by 4 of 5 patients treated at the MTD (Table 2). This toxicity uniformly occurred 16 to 20 h into the infusion and persisted for 12 to 24 h after the end of the infusion. Pain was not alleviated by pretreatment with nonsteroidal antiinflammatories (ibuprofen, indomethacin) and required treatment with narcotic analgesics. By 24 h after the completion and treatment, narcotics were no longer required, and pain abated without specific intervention. No evidence of either cardiac or pulmonary infarction was detected by serial chest X-rays, electrocardiographs, and cardiac enzymes, arterial blood gases, or technetium cardiac scanning. Nausea and vomiting were first reported by patients receiving a dose of 5000 mg/sq m but were never dose limiting. At the MTD, only one of 5 patients developed Grade 3 gastrointestinal toxicity, but in other patients who developed nausea and vomiting at the MTD, symptoms could be controlled by phenothiazine antiemetics.

No other toxicities were observed. Specifically, there was no evidence of myelosuppression, hepatic toxicity, allergic reaction, hypotension, or nephropathy.

Clinical Responses. Dramatic (>50%) reductions in palpable adenopathy were observed in 2 patients with heavily pretreated diffuse histiocytic lymphoma who both received DHAC at the MTD. However, in both of these patients, disease progressed prior to the initiation of Cycle 2, precluding further therapy. In addition, a patient with progressive Hodgkin's disease refractory to 4 different combination chemotherapeutic regimens stabilized for 7 months on single-agent DHAC.

***In Vitro* Stability.** DHAC was unstable in human plasma when it was incubated at the clinically achievable concentrations of 4.5 or 10 μg/ml (Chart 2). The addition of THU such that the plasma concentration was 1×10^{-4} M or 1-β-D-ribofuranosyl-1,3,4,7-tetrahydro-2*H*-1,3-diazepin-2-one at 2×10^{-5} M completely inhibited this decomposition. Accordingly, the more potent diazepinion riboside (17) was added to all sample tubes prior to specimen collection to inhibit cytidine deaminase-mediated decomposition.

Pharmacokinetics. Chart 3 shows a typical C_p *versus* time curve for a patient treated with a 24-h constant-rate infusion of DHAC. Plasma steady-state concentrations are reached within approximately 8 h and are proportional to dose (Table 3). At the termination of the infusion, C_p decays in an apparent biphasic manner with a plasma elimination half-life of 1 to 2 h. Table 3 summarizes the pharmacokinetic parameters for the 9 patients for whom DHAC plasma levels were measured.

DHAC was also measured in the urine of 7 patients (Table 4).

Table 2
Toxicity of DHAC

Dose (mg/sq m)	No. of patients/cycles	Nausea (grade)[a]			Chest pain (grade)[b]		
		1	2	3	1	2	3
1000	2/6	0	0	0	0	0	0
2000	3/4	0	0	0	0	0	0
5000	3/10	0	2	1	0	1	1
7000	5/6	2	3	1	0	1	5

[a] Grade 1, nausea only; Grade 2, transient vomiting; Grade 3, vomiting requiring treatment.
[b] Grade 1, chest discomfort not requiring treatment; Grade 2, pain relieved by narcotic analgesia; Grade 3, chest pain decreased but not completely relieved by narcotic analgesia.

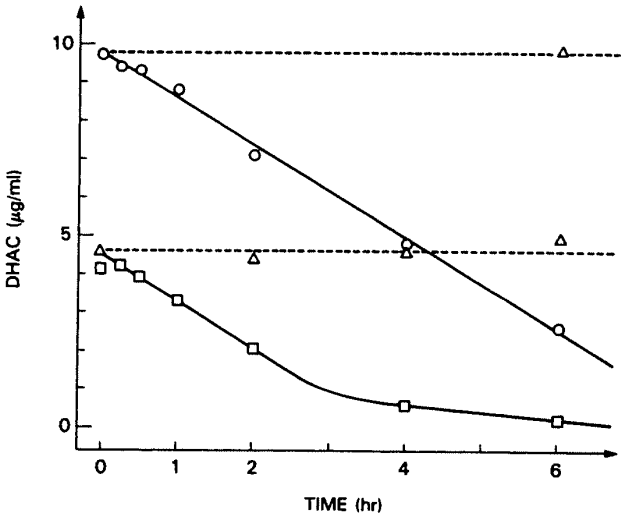

Chart 2. Stability of DHAC in human plasma at 37°C. Fresh human plasma was spiked with DHAC hydrochloride at either 4.5 μg/ml (1.6×10^{-5} M) or 10 μg/ml (3.5×10^{-5} M) and incubated as indicated in "Materials and Methods." *Solid lines* (○, □) depict plasma decomposition in the absence of cytidine deaminase inhibitors. *Dotted lines* represent the corresponding plasma concentrations in the presence of 2×10^{-5} M diazepinone riboside, NSC 322096 (△). Both the diazepinone riboside and THU inhibited *in vitro* decomposition at both DHAC concentrations; the data obtained for 1×10^{-4} M THU were superimposable with those shown for NSC 322096.

Between 8.0 and 20.5% of the administered dose of DHAC could be recovered unchanged in the urine. The vast majority of unchanged drug was excreted during the infusion period with only 1 to 4% appearing after treatment had stopped. Although the data for DHAC urinary excretion are limited and skewed toward the highest doses, there does not seem to be any correlation of the percentage of drug excreted with dose.

DHAC levels were also measured in ascites samples obtained from Patient 6, who had ovarian cancer metastatic to the peritoneum and diaphragm. Comparable levels of drug were seen in the plasma and ascites at the end of the infusion, with concentrations of 20.5 and 20.4 μg/ml, respectively, being measured. However, DHAC removal from the ascites was slower, since by 4 h postinfusion, a 1.81-μg/ml plasma concentration was attained, while the ascites level was more than twice as high at 4.06 μg/ml.

DISCUSSION

DHAC is the dihydro analogue of 5-AC, a nitrogen isostere of cytidine. Although Phase I studies with 5-AC demonstrated objective responses in patients with breast cancer, carcinoma of the colon, and melanoma (20), tolerance to bolus therapy was limited by acute toxicities (nausea, vomiting, diarrhea, fever, and hypotension), while more prolonged infusional therapy was limited by drug instability. Thus, the clinical use of 5-AC has been restricted to acute myelogenous leukemia where consistent antitumor activity has been documented in patients with heavily pretreated disease (4). DHAC was specifically synthesized to preclude drug decomposition in solution via opening of the triazine ring at the hydrolytically susceptible 5,6-imino bond. As predicted by design, DHAC is highly stable in solution. When

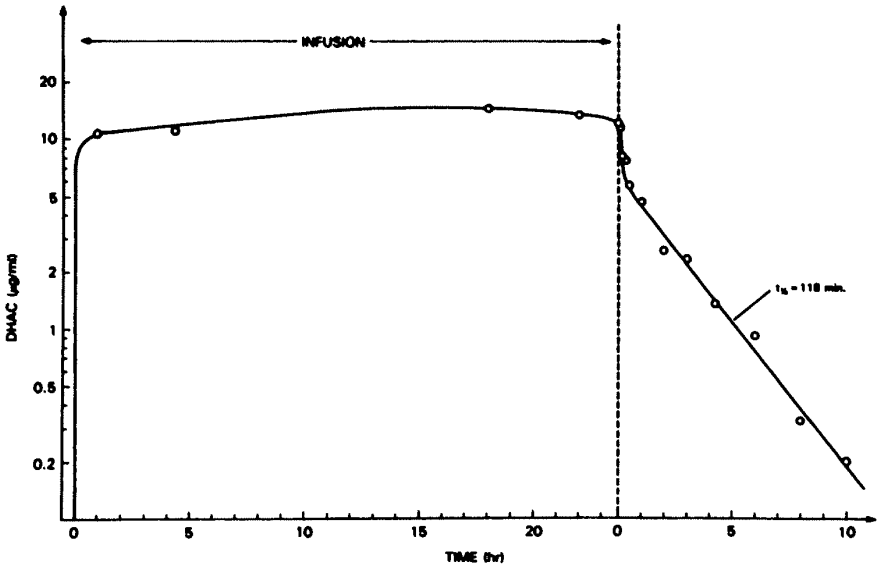

Chart 3. DHAC plasma concentrations in Patient 5 during and after a 24-h infusion of DHAC, 7000 mg/sq m. The *dashed vertical line* indicates the end of the infusion and zero time for the postinfusion period. The *postinfusion curve* defines the 2-compartment open model that best fits the data.

Table 3

Pharmacokinetic parameters of DHAC

Patient	Dose (mg/sq m)	C_{ss} (μg/ml)	CL_{TB}[a] (ml/min/sq m)	$t_{1/2\beta}$ (min)
1	1000	2.62	265	
2	5000	13.6	255	79
3	5000	9.06	383	73
4	5000	13.4	259	62
5	7000	14.6	333	118
6	7000	20.5	237	120[b]
7	7000	18.8	259	
8	7000	10.0	486	
9	7000	15.3	318	86
			311 ± 76[c]	90 ± 22

[a] CL_{TB}, apparent total-body clearance; $t_{1/2\beta}$, plasma elimination half-life.
[b] Estimated from only 3 postinfusion points.
[c] Mean ± SD.

Table 4

Urinary excretion of DHAC

Patient	Dose (mg/sq m)	% of dose excreted	
		Infusion	Total[a]
4	5000	13.9	14.6
5	7000	11.3	—[b]
6	7000	13.1	14.3
7	7000	17.9	19.5
8	7000	9.2	13.5
9	7000	19.3	20.5
10	7000	8.0	—

[a] Infusion plus posttreatment excretion. Postinfusion urine collections covered a period ranging from 4.5 to 20 h.
[b] No postinfusion urine collection.

formulated as directed and diluted in 5% dextrose and 0.9% sodium chloride solution, the compound shows 1 to 2% decomposition over 2 days at room temperature. Under similar conditions, 5-AC would exhibit 16 to 21% decomposition in only 6 h (9). Because of this enhanced stability, DHAC could be administered as a constant infusion over 24 h to patients in this Phase I study.

It was originally hypothesized that DHAC functioned as a prodrug, being converted to 5-AC *in vivo*. This was suggested by studies demonstrating cross-resistance in 5-AC-resistant L1210 (11). However, preclinical pharmacological studies failed to demonstrate any *in vivo* conversion to 5-AC. DHAC was extensively metabolized in the rat following bolus injection with only 30% being excreted unchanged in urine (21). Neither 5-AC nor its principal hydrolysis products were detected by HPLC, and subsequent gas chromatography-mass spectroscopy analysis identified only the deamination products 5,6-dihydro-5-azauracil, 5,6-dihydro-5-azauridine, ammelide, and cyanuric acid as the principal metabolites. Similarly, our studies demonstrate that DHAC is extensively metabolized in humans during and following constant i.v. infusion over 24 h; 8 to 19% of the administered dose was excreted unchanged in the urine over the course of the 24-h infusion. Postinfusion urine did not account for more than an additional 4% of the total dose. DHAC is an excellent substrate for cytidine deaminase (22), and the fact that the drug is deaminated in humans is confirmed by inhibition of DHAC decomposition by adding diazepinone riboside (NSC 322096) to patient samples. Indeed, DHAC plasma stability appears to be related to levels of this enzyme even though relatively little cytidine deaminase is found in plasma. For BALB/c × DBA/2 F_1 mice, which have practically no plasma cytidine deaminase and very low overall levels, an *in vitro* plasma half-life of about 42 h was observed (23). This contrasts with the 4-h half-life observed in this study for human plasma, which has much higher levels of cytidine deaminase (16).

The dose-limiting toxicity of DHAC, pleuritic chest pain, is an unusual toxic end point (24), and the clinical presentation and pharmacokinetic parameters suggest that it may not be a direct drug effect. No correlation was observed between plasma DHAC blood levels and the intensity of the chest pain. Moreover, while steady-state plasma levels were achieved within 8 h, chest pain was not reported by any patient until 16 to 20 h into the infusion; and while the postinfusion half-life of drug was 1 to 2 h, chest pain persisted for as long as 24 h after the end of treatment. These data suggest that this toxicity might be mediated by a metabolite.

Encouraging clinical activity was observed during this Phase I study with 2 patients with diffuse histiocytic lymphoma demonstrating >50% reductions in palpable adenpathy after treatment at the MTD. However, disease progression in both patients prior to initiation of Cycle 2 suggests that DHAC may need to be administered more frequently than every 28 days. In addition, an extensively pretreated patient with progressive Hodgkin's disease stabilized for 7 months at a DHAC dose of 5 g/sq m.

In summary, DHAC is an analogue of 5-AC that was synthesized to overcome the aqueous instability of the parent compound and allow precise dosing with infusional therapy. The drug is extensively metabolized in humans and is rapidly deaminated in plasma; less than 20% of the administered dose is excreted unchanged in urine. The recommended dose for Phase II studies with DHAC using a 24-h infusion is 7 g/sq m. Priority should be given to those diseases in which the parent compound has reproducible antitumor activity.

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Methylation mediated silencing of *TMS1/ASC* gene in prostate cancer

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Abstract

Background: Transcriptional silencing associated with aberrant promoter methylation has been established as an alternate pathway for the development of cancer by inactivating tumor suppressor genes. *TMS1* (Target of Methylation induced Silencing), also known as *ASC* (Apoptosis Speck like protein containing a CARD) is a tumor suppressor gene which encodes for a CARD (caspase recruitment domain) containing regulatory protein and has been shown to promote apoptosis directly and by activation of downstream caspases. This study describes the methylation induced silencing of *TMS1/ASC* gene in prostate cancer cell lines. We also examined the prevalence of *TMS1/ASC* gene methylation in prostate cancer tissue samples in an effort to correlate race and clinico-pathological features with *TMS1/ASC* gene methylation.

Results: Loss of *TMS1/ASC* gene expression associated with complete methylation of the promoter region was observed in LNCaP cells. Gene expression was restored by a demethylating agent, 5-aza-2'deoxycytidine, but not by a histone deacetylase inhibitor, Trichostatin A. Chromatin Immunoprecipitation (ChIP) assay showed enrichment of MBD3 (methyl binding domain protein 3) to a higher degree than commonly associated MBDs and MeCP2. We evaluated the methylation pattern in 66 prostate cancer and 34 benign prostatic hyperplasia tissue samples. *TMS1/ASC* gene methylation was more prevalent in prostate cancer cases than controls in White patients (OR 7.6, p 0.002) while no difference between the cases and controls was seen in Black patients (OR 1.1, p 0.91).

Conclusion: Our study demonstrates that methylation-mediated silencing of *TMS1/ASC* is a frequent event in prostate cancer, thus identifying a new potential diagnostic and prognostic marker for the treatment of the disease. Racial differences in *TMS1/ASC* methylation patterns implicate the probable role of molecular markers in determining in susceptibility to prostate cancer in different ethnic groups.

Background

Gene silencing associated with aberrant promoter methylation has been suggested as an alternate pathway for development of cancer [1]. This form of epigenetic change contributes to tumor initiation and progression by transcriptional silencing of tumor-suppressor genes. Several genes have been shown to be epigenetically inactivated in a wide range of tumors [2] and most neoplasms show hypermethylation of one or more genes [3-5]. This has led to the concept of a 'hypermethylation profile' of tumors which could have potential clinical applications [5-7]. The hypermethylated genes can be broadly classified as those involved in cell cycle regulation (p16INK4a, p15, Rb), genes associated with DNA repair (BRCA1, MGMT), apoptosis (DAPK), and drug detoxification (GSTPi), drug resistance (MGMT), cellular differentiation, angiogenesis (THBS1) and metastasis (E-cadherin) [2].

Epigenetic dysregulation of an apoptotic pathway appears to be connected to the development of many cancers as confirmed by several research communications [8,9]. Apoptosis or 'programmed cell death' is essential for embryonic development and also plays an important role in the immune system and maintenance of cellular homeostasis [10]. A defect in apoptosis is implicated in neurodegenerative diseases, autoimmunity, and cancer and in chemo-resistance. Multiple genes direct the apoptotic pathway to restrain the inappropriate proliferation of cells and a defect in the signaling mechanism gives the cancer cells an added survival advantage leading to tumor initiation, progression and even drug resistance. Apoptosis is mediated by a family of cystine proteases called caspases. There are two groups of caspases – the initiator caspases (CASP8, CASP9 and CASP10) and the effector caspases (CASP3, CASP6 and CASP7). Caspases exist as latent proenzymes and are activated by proteolytic cleavage. Several key genes involved in apoptosis have been showed to be target of epigenetic changes [9].

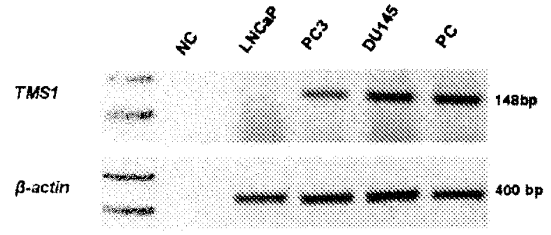

Figure 1
Expression pattern of *TMS1/ASC* gene. Reverse transcription PCR analysis showing expression of *TMS1/ASC* gene in prostate cancer cell lines. β-actin was used as a control for RNA integrity. **NC**-negative control, **PC**-positive control.

Recently, *TMS1* was shown to be aberrantly hypermethylated in breast cancer tissues and cell lines [11]. This gene is also known as ASC (Apoptosis Speck Like protein containing a CARD) [12]. *TMS1/ASC* encodes a 22-kDa CARD protein and promotes apoptosis in a caspase 9 dependent pathway. *TMS1/ASC* has been shown to bind with various proteins like Pyrin, Ipaf and copyrin/PYPAF1[12]. In addition to its role in cancer development there is ample evidence which suggests that *TMS1/ASC* is involved in immune responses and NF-κB and caspase1 activation [13,14]. The downregulation of *TMS1/ASC* in breast cancer cell lines correlates with dense methylation on the CpG islands. Methylation of the promoter region of *TMS1/ASC* has also been identified in small cell lung cancer and non-small cell lung cancer [15], human glioblastoma [16], ovarian tumors [17,18], colorectal cancer [19], neuroblastoma [20], and melanoma [21]. However in a study by Roman-Gomez et. al., on acute lymphoblastic leukemia patients no correlation was found with methylation of the *TMS1/ASC* gene [22].

So far no studies have been reported on the role of *TMS1/ASC* in prostate cancer. In this study we examined the mechanism of *TMS1/ASC* gene silencing in prostate cancer cell lines. We also studied the prevalence of *TMS1/ASC* gene methylation in prostate cancer tissue samples as well as the association of race and clinico-pathological features with *TMS1/ASC* gene methylation.

Results

Expression and methylation status of TMS1/ASC gene in prostate cancer cell lines

By RTPCR (reverse transcriptase polymerase chain reaction), we first analyzed the expression status of *TMS1/ASC* in three prostate cancer cell lines – LNCaP, PC3 and DU145 by RT-PCR. LNCaP exhibited complete loss of *TMS1/ASC* transcript whereas partial expression was detected in the remaining two cell lines (Fig 1). β-actin was used as control for RNA integrity and loading.

Methylation-mediated deregulation of *TMS1/ASC* has been described in breast cancer and in other tumors. To determine if methylation was responsible for down regulation of *TMS1/ASC* in prostate cancer cell lines, we performed MS-PCR (methylation specific PCR) using both methylated and unmethylated primers (Fig 2C). Complete methylation of the promoter region was seen in LNCaP cells whereas PC3 and DU145 showed partial methylation of the *TMS1/ASC* gene (Fig 2D). *TMS1/ASC* expression inversely correlated with the methylation status. To confirm our MS-PCR findings we performed bisulfite genomic sequencing of the sodium bisulfite modified DNA in the three cell lines. In LNCaP cell line the cytosines in the non-CpG sites were converted to thymidine but the cytosines associated with CpG islands

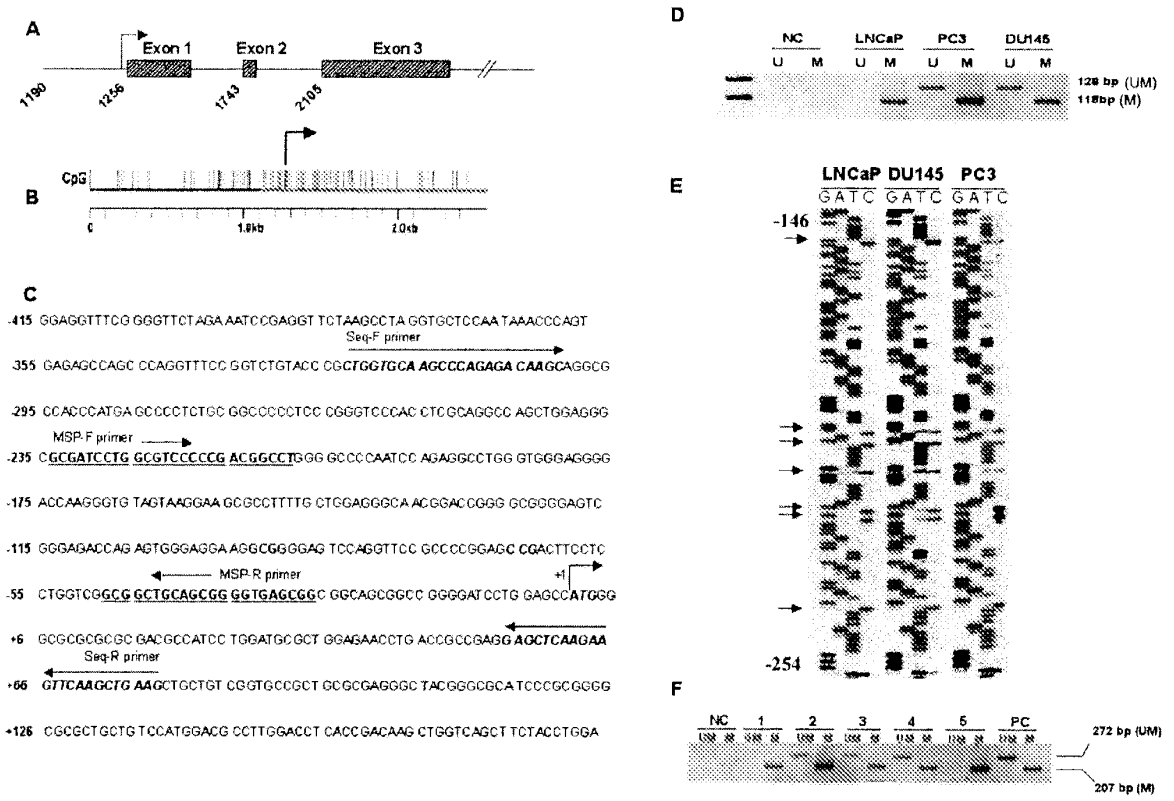

Figure 2

Methylation pattern of *TMS1/ASC*. *Panel A* – graphical representation of the structure of *TMS1/ASC* gene showing position of the three exons. *Panel B* shows the location of the CpG dinucleotides. *Panel C* shows the postion of the primers used for MS-PCR and bisulfite sequencing in the promoter region of *TMS1/ASC*. Positions are indicated relative to translation start site. *Panel D* shows MS-PCR analysis of *TMS1/ASC* gene on the different prostate cancer cell lines. LNCaP exhibits complete methylation of *TMS1/ASC* gene. PC3 and Du145 have both the methylated as well as the unmethylated allele and are consequently expressed. **NC**-negative control, **U**-unmethylated allele, **M**-methylated allele. The methylation pattern correlates with the bisulfite genomic sequencing shown on *panel E*. Completely methylated cytosines (black arrow) in LNCaP are not converted to thymidine following bisulfite treatment and show up on the C lane whereas partially methylated cytosines in the CpG's are seen both in the T lane as well as in the C lane in PC3 and Du145 cell lines. Positions are indicated relative of the translation start site. Panel F shows representative examples of 5 prostate cancer tissue samples analyzed by MS-PCR and gel electrophoresis. Presence of a band in lanes marked as **UM** indicates presence of unmethylated allele and a band in the lanes marked **M** denotes a methylated allele; **NC**-negative control; **PC**-positive control.

remained unmodified after bisulfite treatment thereby demonstrating complete methylation. PC3 and DU145 showed a mixed pattern of methylated as well as unmethylated cytosine in the CpG dinucleotides (Fig 2E). We also performed MS-PCR on DNA extracted from 5 representative tissue samples of prostate cancer patients. Two of the samples showed complete methylation, while the remaining three showed partial methylation of *TMS1/ASC* gene promoter (Fig 2F).

5-Aza-2'-deoxycytidine and TSA treatment of LNCaP cell line

We treated LNCaP cells with 5-Aza-2'-deoxycytidine in an attempt to induce *TMS1/ASC* gene expression. 5-Aza-2'-deoxycytidine is a demethylating agent and forms a covalent complex with DNA methyltransferase thus inhibiting DNA methylation. The *TMS1/ASC* gene expression was examined by RT-PCR analysis and methylation status of *TMS1/ASC* gene examined by MS-PCR of the bisulfite modified drug treated DNA. Following 5-Aza-2'-deoxycytidine treatment the *TMS1/ASC* gene underwent partial

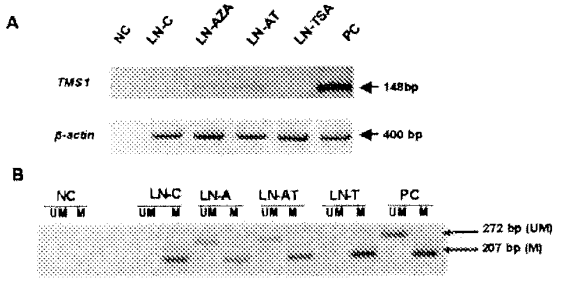

Figure 3
Expression and methylation pattern of *TMS1/ASC* gene in drug treated LNCaP cells and in Tumor tissues. *Panel A* shows restoration of *TMS1/ASC* gene expression following treatment with 5-Aza but not with TSA alone. *Panel B* – MS-PCR analysis on the drug treated LNCaP cells confirm expression of *TMS1/ASC* is associated with partial demethylation of the gene seen as a band on the UM lane in 5-Aza treated cells. Presence of a band in lanes marked as **UM** indicates presence of unmethylated allele and a band in the lanes marked **M** denotes a methylated allele. **NC**-negative control; **PC**-positive control; **UM**-unmethylated allele; **M**-methylated allele; **LN-C**-untreated LNCaP cells; **LN-AZA** -5-AZA-2'-deoxycytidine treated cells; **LN-AT** – 5-AZA-2'-deoxycytidine followed by TSA; **LN-TSA** – TSA treated cells.

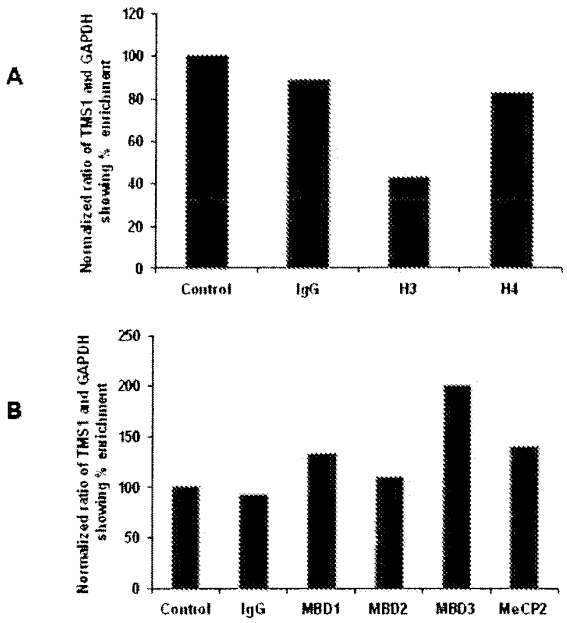

Figure 4
Binding pattern of acetylated histones (*panel A*) and MBD's (*panel B*) to the methylated *TMS1/ASC* gene. Chromatin immunoprecipitation performed on formalin fixed and sonicated LNCaP chromatin shows enrichment of MBD3 in the immunoprecipitated DNA and low levels of acetylated H3 suggesting deacetylation of H3 in the silenced TMS1/ASC gene.

demethylation and resulted in expression of the gene (Fig 3). Since methylated DNA binds to methylcytosine binding proteins which in turn interact with histone deacetylases (HDAC), we treated the LNCaP cells with a histone deacetylase inhibitor TSA to see if it can induce expression of the *TMS1/ASC* gene. TSA treatment failed to induce *TMS1/ASC* mRNA expression in LNCaP cells (Fig 3A). Methylation pattern analysis of drug treated cells showed that *TMS1/ASC* promoter remained fully methylated in TSA-treated cells whereas both unmethylated and methylated alleles exist in 5-AZA-2'-deoxycytidine treated cells (Fig. 3B).

Histone acetylation and interaction of methylcytosine binding proteins with TMS1/ASC promoter in LNCaP cell line

Recent studies have shown that deacetylation of histone H3 and H4 by the HDAC's presumably leads to the formation of a chromatin environment that inhibits transcription. To determine if methylation induced silencing of *TMS1/ASC* is associated with changes in histone acetylation pattern we studied the pattern of histone acetylation at the *TMS1/ASC* promoter in LNCaP cells using ChIP assay. Neither acetylated H3 nor acetylated H4 binds to *TMS1/ASC* promoter region in LNCaP (Fig 4A). Since methylcytosine binding proteins mediate the repressive effect of methylated DNA, we examined the interaction of methylcytosine binding proteins with the *TMS1/ASC* promoter. The TMS1 promoter interacted mainly with MBD3 though minimal binding of MBD1, MBD2 and MeCP2 at *TMS1/ASC* promoter was also seen (Fig 4B).

Patient characteristics and association with TMS1/ASC gene methylation

Table 2 shows the demographics and clinical characteristics of the study subjects. The prostate cancer patients (51 – 80 yrs) were 7 years younger than BPH (53–88 yrs) on an average. However the racial distribution was similar. Most of the patients were stage 2–3 and had Gleason score of 6–7. Table 3 shows the frequency of *TMS1/ASC* gene methylation in all the samples examined. *TMS1/ASC* gene methylation was seen in 63.6% of prostate cancer but in only 35.3% of BPH patients with an age-adjusted odds ratio of 3.7 (95% CI 1.4 – 9.4). The prevalence of *TMS1/ASC* gene methylation in prostate cancer patients was found to be similar in both races (66.7% in Blacks as compared to 62.2% in Whites). However comparing the prevalence of *TMS1/ASC* methylation between prostate-cancer patients and controls, there was no difference among

Table 1: Table showing details of primers used and cycling conditions for MS-PCR, RT-PCR and PCR amplification of the CHIP DNA.

Gene	*Primer sequence (5'-3')*	*Anneal. Temp. (°C)*	*No. of Cycles*
TMS1/ASC methylated	F- CGA TTT TGG CGT TTT TCG ACG GTT R- CCG CTC ACC CCG CTA CAA CCG C	65	35
TMS1/ASC Unmethylated	F- TTG TTG GAG GGT AAT GGA TT R- CCC ACA AAA ATA CAC CCA TA	58	35
TMS1/ASC RT-PCR	F- GGA CGC CTT GGC CCT CAC CG R- GGC GCG GCT CCA GAG CCC TG	65	35
TMS1/ASC CHIP	F- GAG TCG GGA GAC CAG AGT GGA R- ACA GCA GCT TCA GCTT GAA CTT CTT G	68	50
β-actin	F- ACC ATG GAT GAT GAT ATC GC R- ACA GGC TGG GGT GTT GAA G	60	30
GAPDH	F- CCC CAC ACA CAT GCA CTT ACC R- CCT AGT CCC AGG GCT TTG ATT	65	50

Blacks (prevalence 66.7% for patients and 58.3% for controls), whereas in Whites a significant difference was observed (prevalence 62.2% for prostate cancer cases and 22.7% for BPH controls). The age adjusted odds ratio comparing cases to controls among White patients was 7.6 (p = 0.002). We also studied the effect of age and *TMS1/ASC* gene methylation but no association with early or late onset prostate cancer was observed (data not shown). No association with stage of the disease was observed but there was a tendency of *TMS1/ASC* gene methylation to be more frequent in tumors having Gleason score 7 or higher. (Table 4)

Table 2: Characteristics of 66 prostate-cancer patients and 34 BPH controls.

	Prostate cancer		Control (BPH)		
	n	%	n	%	P-value
Race					
Black	21	31.8	12	35.3	0.82
White	45	68.2	22	64.7	
Stage					
II	42	63.6			
III	19	28.8			
IV	4	6.1			
Unknown	1	1.5			
Gleason Score					
5	3	4.6			
6	26	39.4			
7	32	48.4			
8	3	4.6			
9	2	3.0			
Preoperative serum PSA					
< 4	5	7.6			
4.0 – 8	46	69.7			
8.1 – 12	8	12.1			
> 12	7	10.6			
Mean age (range)	64 yrs (51 – 80)		71 yrs (53 – 88)		< 0.001

Discussion

Our study shows a clear correlation between *TMS1/ASC* methylation and silencing of the gene suggesting a role in prostate cancer cell lines. Re-expression accompanied by partial demethylation of *TMS1/ASC* following 5-AZA-2'-deoxycytidine confirms that methylation is responsible for transcriptional silencing of this gene. Lack of response to TSA treatment suggests that histone acetylation does not play a role in downregulating the expression of *TMS1/ASC*. This finding is similar to the results obtained by Stimson et al[23]. Over expression of *TMS1/ASC* was shown to inhibit cellular proliferation and induce DNA fragmentation which can be blocked by a caspase inhibitor [24]. In addition, forced reduction in *TMS1/ASC* promotes cell survival perhaps in a NFκ-B dependent pathway [25]. This makes it a therapeutic target by use of demethylating agents alone or in combination with additional apoptosis inducing drugs.

ChIP analysis showed binding predominantly with MBD3 and only minimal enrichment of the other MBDs and MeCP2. Earlier studies have demonstrated that MBD3 does not bind to methylated DNA alone [26,27]. However, it has a definite role in maintaining methylation. Recent studies have shown binding of MBD3 to several genes like cox6c, leng6, bat5 etc. [28]. Wade et al showed that MBD3 is a subunit of the NuRD complex that has nucleosome remodeling and histone deacetylase activities [29]. MBD3 forms a part of the multiprotein NuRD complex and probably has a role as a transcriptional co-repressor. Our finding that MBD3 binds to methylated *TMS1/ASC* is contrary to the known pattern of MBD binding by MBD proteins to methylated DNA. This could be because MBD3-containing NuRD complexes bind more specifically to the methylated *TMS1/ASC* gene [28].

A noteworthy finding in our study was the statistically significant difference in the methylation of *TMS1/ASC*

Table 3: Overall and race-specific frequencies of *TMS1/ASC* gene methylation in prostate-cancer patients and BPH controls, with age-adjusted odds-ratio estimates of the relative risk of prostate cancer associated with *TMS1/ASC* gene methylation.

Patients	Number and (%) of tissues with *TMS1/ASC* methylation Prostate Cancer	Control (BPH)	Age-adjusted odds ratio* (95% CI)	P-value
All	42/66 (63.6)	12/34 (35.3)	3.7 (1.4 – 9.4)	0.008
Blacks	14/21 (66.7)	7/12 (58.3)	1.1 (0.2 – 5.5)	0.91
Whites	28/45 (62.2)	5/22 (22.7)	7.6 (2.1 – 27.3)	0.002

* For the overall odds ratio estimate, we modeled the logit of the probability of being a case (p), as a function of *TMS1/ASC* gene methylation and age: $\ln [p/(1-p)] = b_0 + b_1 \times TMS1/ASC + b_2 \times Age$. For the race-specific age-adjusted odds-ratio estimates, the following model was fitted: $\ln [p/(1-p)] = b_0 + b_1 \times TMS1/ASC + b_2 \times Age + b_3 \times Race + b_4 \times TMS1/ASC \times Race$. The interaction term *TMS1/ASC*Race was marginally significant (p = 0.068).

among the cases and controls in Whites compared to Blacks. We observed an age adjusted odds-ratio of 7.6 (95% CI 2.1–27.3) in Whites as compared to only 1.1 (95% CI 0.2–5.5) among Blacks. Whether this finding reflects involvement of different pathogenetic pathways in different races will be interesting to study. In the US, the incidence and mortality of prostate cancer is about two-fold higher among Blacks compared to Whites, suggesting racial differences in prostate tumor occurrence and aggressiveness [30]. The reason for these racial differences is not well understood. It is possible that promoter-region gene hypermethylation may be influenced by environmental exposures.

There have been only a few studies on differences in gene methylation between different races. Two studies by Woodson et al showed a differential methylation pattern and expression of CD44 in Blacks and Whites [31,32]. When we examined 5 genes frequently methylated in prostate cancer (GSTP1, CD44, ECAD, RASSF1A and EBR) in the same patient population we did not find any significant differences in the gene specific methylation pattern between the different races (Table 5). Ethnic group related differences in hypermethylation of promoter region of GSTP1 gene were reported in a recent paper [33]. The authors observed higher hazard ratio (HR) for pathogenesis among African Americans as compared to Caucasians. In contrast to the above observation, we found the hazard ratio of *TMS1/ASC* methylation (prostate cancer versus BPH) to be lower in Blacks as compared to Whites. Ethnic origin is an important determinant of prostate cancer risk, incidence, and disease progression. In the US, the African-American male group has the highest incidence rate for prostate cancer [30]. Differences in diet, socioeconomic environment, lifestyle between the two ethnic groups have been implicated as causative factors for the striking ethnic differences in the incidence and clinical behavior of prostate cancer. However, molecular mechanisms underlying the racial diversity are not well understood. The recent report by Fang et. al., shows that Genistein leads to reversal of hypermethylation and reactivation of p16INK4a, RARβ, and MGMT genes [34]. Thus, diet seems to be an important factor in affecting the methylation status of different genes implicated in cancer. It is also likely that genes are differentially methylated in different ethnic groups owing to the lifestyle and dietary differences. Methylation of promoter in controls (BPH) may reflect that epigenetic alteration of the gene has already occurred and that they have acquired epigenetic malignant potential even though the pathological diagnosis classifies them as benign [33]. Our results indicate that differences in methylation pattern of *TMS1/ASC* in BPH among ethnic groups might explain the differences among different racial groups in susceptibility to prostate cancer. Thus it seems that the epigenetic make up of different ethnic groups would determine the risk to prostate cancer pathogenesis.

Table 4: Effect of *TMS1/ASC* gene methylation on clinical stage (II or III), and Gleason score (6 or 7).

	Number and (%) of tissues with TMS1/ASC methylation	Age-adjusted odds-ratio* (95% CI)	P-value
Clinical stage			
II (reference)	28/42 (66.7)		
III	11/19 (57.9)	0.7 (0.2 – 2.1)	0.53
Gleason score			
6 (reference)	14/26 (53.9)		
7	23/32 (71.9)	2.3 (0.7 – 7.2)	0.15

* Age-adjusted relative risk estimates of stage III vs. II, or Gleason score 7 vs. 6, derived from logistic model $\ln (p/q) = b_0 + b_1 \times TMS1/ASC + b_2 \times Age$, where p is either the probability of having stage III disease or of having Gleason score 7, and q = (1-p) is the probability of the other category.

Table 5: Risk of prostate cancer in relation to gene methylation, total patients and by race.

Gene	Number and (%) of tissues with gene methylation		Age-adjusted	P-value
	Prostate Cancer	Control (BPH)	odds ratio* (95% CI)	
TMS1/ASC	42/66 (63.6)	12/34 (35.3)	3.7 (1.4 – 9.4)	0.008
Blacks	14/21 (66.7)	7/12 (58.3)	1.1 (0.2 – 5.5)	0.914
Whites	28/45 (62.2)	5/22 (22.7)	7.6 (2.1 – 27.3)	0.002
GSTP1	46/66 (69.7)	1/34 (2.9)	69.7 (8.7 – 558.7)	<0.0001
Blacks	15/21 (71.4)	0/12 (0.0)	--	
Whites	31/45 (68.9)	1/22 (4.6)	--	
CD44	46/66 (69.7)	13/34 (38.2)	2.7 (1.1 – 6.9)	0.033
Blacks	14/21 (66.7)	4/12 (33.3)	2.9 (0.6 – 14.4)	0.190
Whites	32/45 (71.1)	9/22 (40.9)	2.6 (0.9 – 8.1)	0.093
ECAD	39/66 (59.1)	5/34 (14.7)	7.9 (2.6 – 24.0)	<0.001
Blacks	15/21 (71.4)	2/12 (16.7)	10.9 (1.7 – 70.9)	0.013
Whites	24/45 (53.3)	3/22 (13.6)	7.2 (1.8 – 29.2)	0.006
RASSF1A	34/66 (51.5)	6/34 (17.7)	4.1 (1.5 – 11.8)	0.008
Blacks	10/21 (47.6)	1/12 (8.3)	8.6 (0.9 – 84.7)	0.064
Whites	24/45 (53.3)	5/22 (22.7)	3.2 (0.9 – 10.7)	0.056
EBR	49/66 (74.2)	23/34 (67.7)	1.3 (0.5 – 3.5)	0.582
Blacks	15/21 (71.4)	7/12 (58.3)	1.9 (0.4 – 9.5)	0.460
Whites	34/45 (75.6)	16/22 (72.7)	1.1 (0.3 – 3.7)	0.912

* For the overall odds ratio estimate, we modeled the logit of the probability of being a case (p), as a function of methylation in the particular gene and age: $\ln [p/(1-p)] = b_0 + b_1 \times \text{gene} + b_2 \times \text{Age}$. For the race-specific age-adjusted odds-ratio estimates, the following model was fitted: $\ln [p/(1-p)] = b_0 + b_1 \times \text{gene} + b_2 \times \text{Age} + b_3 \times \text{Race} + b_4 \times \text{gene} \times \text{Race}$. The interaction between TMS1/ASC and race was marginally significant (p = 0.068), while the interactions between each other genes and race were not statistically significant at the 5% level (that is, corresponding p-values were greater than 0.454).

There did not appear to be a significant relationship between *TMS1/ASC* methylation status and patient's age. On our limited dataset there was a trend towards association between *TMS1/ASC* methylation and Gleason score 7 or higher (odds-ratio 2.3). Though the relationship was not statistically significant it possibly suggests a worse prognosis as patients with Gleason score 7 do worse that those with Gleason score 6. Many of our patient samples showed partial methylation status – this could be due to presence of normal fibroblasts, endothelial cells, inflammatory cells and non malignant prostate tissue surrounding the tumor. Tumors where *TMS1/ASC* was not found to be methylated could involve other genes in the apoptotic pathway. Use of pathway specific cDNA microarrays may help in determining the individual genes affected.

In summary our study has shown that *TMS1/ASC*, a pro-apoptotic gene, is silenced by hypermethylation of the CpG islands in the promoter region. This transcriptional repression is relieved by treatment with a demethylating agent (5-Aza-2'-deoxycytidine). Frequent methylation of *TMS1/ASC* in prostate cancer suggests this gene may be important in pathogenesis of prostate cancer and can be a target of pharmacologic demethylation in clinical trials. *TMS1/ASC* methylation patterns show significant ethnic differences. Our findings provide a novel insight into the molecular determinants of tumor growth that may underlie the ethnic differences in prostate cancer incidence and clinical behavior. Further studies are needed to find out if this has any significant clinical implications in the development of novel diagnostic approaches for biologically aggressive prostate cancer from diverse racial origin.

Methods

Cell lines

LNCaP, PC3 and DU145 prostate cancer cells, obtained from American Type Culture Collection were maintained in DMEM (Invitrogen, Carlsbad, CA) and supplemented with 10% fetal bovine serum in a humidified incubator at 37°C with an atmosphere of 5% CO_2.

Tissue samples

This IRB-approved study involved patients who either had radical prostatectomy between 1998 and 2002 for prostate cancer or trans-urethral resection between 2000 and 2002 for benign prostatic hypertrophy. Sixty-six prostate cancer and 34 benign prostatic hyperplasia (BPH) patients were selected for the study. The prostate-cancer cases and the BPH controls represented all patients who had archived tissue available for study. The characteristics of these two groups are shown in Table 2. The Whites were of northern European descent and did not include Hispanics

Drug treatment of prostate cancer cell lines

For 5-Aza-2'-deoxycytidine (Sigma, St. Louis, MO) and Trichostatin A (TSA) (Sigma St. Louis, MO) treatment, LNCaP cells were grown in 100 mm dishes and treated with 1 μm 5-Aza-2'-deoxycytidine and 300 nm TSA. 5-Aza-2'-deoxycytidine treatment was continued for 7 consecutive days where as TSA treatment was done for 6 hours on the final day. RNA, DNA and chromatin were extracted on day 7.

RNA extraction and reverse transcriptase PCR (RT-PCR)

RNA was extracted from the cell lines using RNA Stat 60 (Tel-Test Inc. Friendswood, TX) as per the manufacturer's instructions. cDNA was prepared from 10 μg of RNA using the Reverse Transcription System from Promega (Madison, WI) using random primers. Two μl of the cDNA was used for the PCR reaction. The primers and PCR conditions used are mentioned in Table 1. Human beta actin (ACTB) was used as the housekeeping gene for loading control.

DNA extraction, bisulfite treatment and methylation-specific PCR (MS-PCR)

Methylation patterns of the prostate cancer cell lines and the tissue samples were analyzed by MS-PCR of bisulfite treated DNA. DNA was extracted from the prostate cancer cell lines using DNA Stat 60 (Tel-Test Inc. Friendswood, TX.). DNA from archived paraffin blocks was isolated using a QIAmp mini-kit (QIAGEN, CA) as per the manufacturer's protocol. This method successfully isolates DNA suitable for PCR amplification from fixed tissues. Genomic DNA was treated with sodium bisulfite under conditions that converts unmethylated cytosine to uracil while the 5-methylcytosine remains unchanged [35,36]. The bisulfite conversion reaction was carried out by incubating 5 μg DNA with a 5 M bisulfite solution and 100 mM hydroquinone, pH 5.0 at 50°C for 4 hours. This was followed by desulfonation by addition of 3 M NaOH, and desalting using a QIAquick column (Qiagen, CA). MS-PCR was performed using methylated and unmethylated *TMS1/ASC* primers (Accession No. AF184072). The primers described by Virmani et. al., [15] located in the promoter region were used in the study. Details of the primers used are mentioned in Table 1. Human placental DNA was methylated in vitro using *sssI* DNA Methylase (NEB) and bisulfite treated for use as a positive control.

Bisulfite sequencing

Five μl of the bisulfite treated DNA from LNCaP, PC3 and Du145 cell lines was amplified by PCR using the BST sequencing primers. The primers were designed to amplify both methylated as well as unmethylated DNA. The PCR products were run on a 2% agarose gel and the desired band was gel purified using Promega SV gel purification Kit (Promega Corp). The Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit (USB Corp., Cleveland, OH) was used for sequencing using forward and reverse primers. The sequencing gel was dried, and radioactive bands were analyzed using Storm phosphor imager (GE Health Care).

Chromatin Immunoprecipitation (CHIP) and Real time PCR

Chromatin immunoprecipitation assays were carried out with a kit from Upstate Biotechnology (Lake Placid, NY) using the manufacturer's protocol and reagents except that the reactions were scaled down ten-fold. Briefly, 2×10^7 cells were incubated in 0.5% formaldehyde for ten minutes to crosslink bound proteins, washed, lysed in SDS lysis buffer and sonicated to 100–500 bp lengths. Ten μl chromatin was mixed with 90 μl of ChIP dilution buffer and precleared with Protein A agarose, and then the chromatin was incubated with anti acetylated H3 and H4 antibody overnight at 4°C. Thirty μl of Protein A agarose beads was added and the chromatin was immunoprecipitated 2 hours at 4°C. The supernatant (unbound chromatin) and beads (bound chromatin) were separated. The beads were washed five times with the buffers provided and then the chromatin was eluted twice in 1%SDS in 0.1 M $NaHCO_3$. Cross-linking was reversed by adding 5 M NaCl and incubating at 65°C for at least 4 hours, following which proteinase K digestion was carried out and DNA was extracted with phenol/chloroform. DNA was ethanol precipitated and dissolved in 100 μl of water.

Real time PCR was performed in triplicate using 5 μl of the immunoprecipitated DNA using primers for *TMS1/ASC* and GAPDH as a housekeeping gene. Quantitative PCR was carried out in a reaction volume of 25 μl using iQ™ SYBR® Green Supermix (BioRad Laboratories, Hercules, CA) on MyiQ™ Single-Color Real-Time PCR Detection System (BioRad Laboratories, Hercules, CA). The final reaction mixture contained 400 nmol/L of each primer. PCR was done for 50 cycles at an annealing temperature of 68°C for *TMS1/ASC* and 65°C for GAPDH. A standard curve was prepared for both the genes using a serial dilution of sonicated human placental DNA (Sigma Chemical Co.). Appropriate negative controls were included in each run. Ratio of *TMS1/ASC* to GAPDH was calculated for each sample.

Statistical analyses

SAS software (release 8.2, SAS Institute) was used for statistical analyses. Student's t test (Armitage, Berry & Matthews 2001) was used to compare the average age in the BPH and prostate-cancer groups. Logistic regression (Hosmer & Lemeshow, 2000) was used to determine the effect of methylation in the particular gene on prostate cancer risk, whether this effect was similar for Blacks and Whites, and whether *TMS1/ASC* gene methylation was associated

with Gleason score, clinical stage, or age of prostate cancer onset. In particular, since prostatic tumors with Gleason-score values of 7 have a substantially worse clinical course than those with values of Gleason score 6, and since few patients (only 10%) had Gleason-score values other than 6 or 7, we used logistic regression to compare the relative frequency of *TMS1/ASC* methylation in tumors with Gleason score 7 versus 6. For association with clinical stage, we likewise compared *TMS1/ASC* methylation in stage 3 versus stage 2 tumors because these two stages comprised 93% of tumors studied. Since prostate-cancer patients were on average 7 years younger than the BPH controls, all of the odds-ratio estimates of relative risk associated with a particular gene methylation were adjusted for age as a continuous covariate in the logistic regression analyses.

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5-Azacytidine Increases HbF Production and Reduces Anemia in Sickle Cell Disease: Dose–Response Analysis of Subcutaneous and Oral Dosage Regimens

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Varying doses of 5-azacytidine (5-aza) were given to four sickle cell individuals for 500, 200, 100, and 30 days. The percentage of fetal hemoglobin (HbF) containing reticulocytes (F reticulocytes) increased two- to five-fold within five days of 5-aza therapy in all patients, with a two- to three-fold rapid response (<48 hours after initial dose) in three patients. Reticulocyte suppression was not observed prior to, during, or after therapy in those patients who responded within 48 hours. Subcutaneous 5-aza was given in 35-day courses consisting of every day, every other day, or three consecutive days a week. No marrow toxicity was observed on any of the regimens. For three patients, the highest average F reticulocyte level was observed on the three consecutive day a week regimen. Oral 5-aza, given with tetrahydrouridine, produced comparable F reticulocyte response. In the two patients treated for more than 100 days, Hb levels increased to 11 to 12 and 9 g/dL, MCV and MCH increased by 25%, and lysate HbF levels peaked at 12% and 20%. Fetal erythroid characteristics (i-antigen, galactokinase activity, and Gγ/Aγ ratios) did not correlate with maximal HbF production. The frequency of vaso-occlusive crises appeared to decrease in both patients followed for more than 100 days.

5-AZACYTIDINE (5-aza), a cell-cycle–specific DNA hypomethylating agent,[1] has been shown to increase fetal hemoglobin (HbF) production in animals,[2,3] in one individual with thalassemia,[4] and in several individuals with sickle cell (SS) anemia.[5,6] In the first phase of our study[5] of repeated doses of 5-aza in one SS patient treated for 100 days, we observed: (1) a rapid increase (within 48 hours after each dose of 5-aza) in the percentage of reticulocytes containing HbF (F reticulocytes), (2) increase in the percentage of HbF and hemoglobin levels in the peripheral blood without evidence of marrow toxicity; and (3) no apparent decrease in the frequency of vaso-occlusive crisis. Alterations in the pattern of DNA methylation at CpG dinucleotide sequences around the β-globin gene complex were associated with increased HbF production in individuals treated with 5-aza,[4–6] but the mechanism by which 5-aza increased levels of HbF production remained uncertain.

In this article we expand our observations concerning the effect of 5-aza therapy in patients with SS anemia. An optimal dose has been defined as that which produces an average F reticulocyte count of at least 20%, but which leads to minimal marrow and gastrointestinal toxicity. The 20% level was chosen because one of us had shown[7] that Saudi Arabian SS patients with clinically mild disease associated with increased production of HbF had F reticulocyte levels between 20% and 50%. We varied the dose and frequency of subcutaneous (SC) 5-aza administration in order to determine an optimal dose regimen, and then determined an optimal oral regimen of 5-aza administration, prompted by observations that 5-aza was orally effective when given to mice[8] or baboons[9] in conjunction with a cytidine deaminase inhibitor, tetrahydrouridine (THU).

MATERIALS AND METHODS

Patients. All patients were adult homozygous SS patients who had become refractory to chronic transfusion therapy for complications of their disease. Patient A (J.P.) was described previously.[5] Patients B (D.P.), C (W.T.), and D (M.J.) were 26, 45, and 23 years old, respectively. Patient A was treated for 500 days. Patient B was dropped from the study due to noncompliance after day 100. Patient C was treated for 200 days. Patient D discontinued therapy at day 30 because he elected to undergo bilateral hip replacement for preexisting aseptic necrosis of both femoral heads. All patients were treated according to protocols approved by The Johns Hopkins University Joint Committee on Clinical Investigation and gave consent after being informed of the potential carcinogenic,[10] and cytotoxic side effects of 5-aza. All patients were hospitalized during the first 45 days of therapy and during regimens of therapy when 5-aza was given daily or on every other day. The remainder of therapy was accomplished as outpatients with the subjects making visits to the hospital two to three times a week.

Drugs. Injectible 5-aza was obtained from the Division of Cancer Treatment, National Cancer Institute. Purified pyrogen-free 5-aza and THU were provided by Drs J. Posada and P. Davignon of the National Cancer Institute, and stored at −70 °C. Encapsulation of 5-aza and THU was performed by The Johns Hopkins Hospital Pharmacy, using precautions suitable for chemotherapeutic agents. Claimed exemptions for new drugs (INDs) were filed with the Food and Drug Administration for all phases of the study. For SC injection, 100 mg of injectible azacytidine was suspended in 4 mL of water for injection and injected as a slurry.

HbF levels. The percentage of F reticulocytes and the amount of HbF/F cell were determined using polyclonal rabbit anti-human HbF.[11,12] The percentage of mature erythrocytes containing HbF (percentage of F cells) was assayed using a mouse monoclonal anti-human HbF antibody developed in our laboratory. Lysate HbF levels were measured by alkali denaturation[13] and Gγ/Aγ ratios were kindly measured by Dr Blanche Alter[14] and Dr Walter Schroeder.[15]

RBC indices were measured by an electronic cell counter that had been standardized with blood collected in K3EDTA. Twenty-step phthalate ester density gradients, supplied by Dr C. Noguchi, were used to measure the mean corpuscular hemoglobin concentration (MCHC) and the percentage of dense cells (MCHC > 37 g/dL).[16,17]

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Vasoocclusive crises. Vasoocclusive crises were defined as episodes of localized pain associated with tachycardia, fever, an elevated WBC, or an increase in total bilirubin. Only one painful episode in patient A was not associated with such findings.

Miscellaneous. Activity of galactokinase was measured by Dr E. Beutler.[18] Erythrocyte folate, serum B-12, α-fetoprotein, and carcinoembryonic antigen levels were measured in The Johns Hopkins Hospital Clinical Laboratory using standard methods. RBC i-antigen titers were measured by a standard agglutination technique[19] using antisera kindly provided by Dr M. Crookston. Cytidine deaminase levels[20] were measured in peripheral blood mononuclear cells separated on Percoll.[21] DNA polymorphism haplotypes were determined by restriction enzyme analysis of peripheral blood by Dr S. Antonarakis.[22]

RESULTS

Onset of F reticulocyte response. Figure 1 indicates that the rapid increase in percentage of F reticulocytes described previously for patient A (5) occurred in three of four patients treated with 2 mg/kg/d SC 5-aza given as a single dose for three consecutive days. In the three patients exhibiting a rapid response, total reticulocyte levels remained at pretherapy levels during this period. It is unclear why patient D responded more slowly. Only he exhibited nausea and vomiting and a decrease in reticulocyte production (2.9 to 5.3 $\times$ $10^5/\mu$L pretherapy to 1.5 $\times$ $10^5/\mu$L ten days posttherapy) at this dosage level.

Effect of various 5-aza doses on F reticulocyte production. Table 1 summarizes the mean F reticulocyte responses of patients A, B, and C given various doses of 5-aza. Assays were performed two to three times a week during each period of treatment. Drug regimens were changed at times when F reticulocyte levels had returned to pretreatment levels. Note that, as described in baboons by DeSimone,[23] baseline F reticulocyte levels seemed to influence the response to treatment. On all dose schedules, the mean F reticulocyte level achieved for patients A and B (pretreatment levels 4.4% $\pm$ 1.0% and 3.8% $\pm$ 0.8%, respectively) was always less than that in patient C (pretreatment level, 14.4% $\pm$ 1.2%). None of the drug regimens listed in Table 1 caused marrow toxicity (defined by decreased WBC, platelet, or reticulocyte counts compared to pretherapy values), even after administration of 5-aza for 30 consecutive days at 2 mg/kg/d (regimen I). In subject A, administration of 5-aza for three consecutive days each week (regimen III) resulted in F reticulocyte levels comparable to those obtained with daily therapy at the same dose. Alternate-day administration (regimen II) or divided daily doses (regimen IV) were less effective than regimen III.

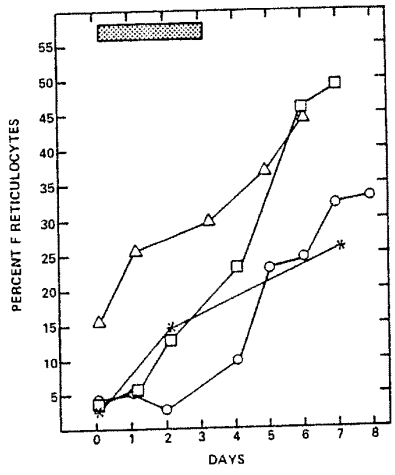

Fig 1. The F reticulocyte response in four sickle cell (SS) patients given subcutaneous (SC) 5-azacytidine (5-aza). Patient A, □; patient B, •; patient C, △; patient D, ○. Note that total reticulocyte counts were unchanged during this period of time in all patients except for patient D (see Results). Shaded bar, top of the graph, refers to days of administration of 5-aza, 2 mg/kg/d (SC) for three consecutive days. Data on patients A, B, and C have been previously described.[27]

Table 1. Average F Reticulocyte Response to Various Doses of Subcutaneous (SC) and Oral (PO) 5-Azacytidine

	Patients		
	A	B	C
Pre	4.4 ± 1.0* (0.2)†	3.8 ± 0.8 (0.2)	14.4 ± 1.2 (0.5)
I	25.0 ± 5.6 (1.2)		
II	17.6 ± 2.7 (0.9)		
III	26.1 ± 3.5 (1.3)	21.9 ± 7.3 (1.0)	31.6 ± 8.7 (1.1)
IV	16.8 ± 7.4 (1.0)	17.8 ± 5.6 (0.9)	
V	26.3 ± 10.0 (1.0)		31.9 ± 10.0 (1.0)

*Mean ± 1 SD percentage of F reticulocytes.

†Mean absolute F reticulocyte level $\times$ $10^6/\mu$L.

I, 2 mg/kg/d for 35 days (SC); II, 2 mg/kg/every other day for 35 days (SC); III, 2 mg/kg/d for three consecutive days per week for five weeks (SC); IV, 2 mg/kg/d for three consecutive days per week for five weeks (SC) 2 doses per day; V, 0.2 mg/kg/d for three consecutive days per week (PO) + 200 mg THU (PO) for five weeks.

Oral administration of 5-aza with THU (regimen V) in patient A and C increased F reticulocyte production, although oral doses of 5-aza (2 mg/kg/d) or THU (200 mg/d) given alone did *not* (data not shown). THU (1.5 to 2 mg/kg) was given one hour before 5-aza, and an equal second dose was given with 5-aza; no food was given until one hour after the second dose. When 5-aza, 2 mg/kg/d, was given with THU for three successive days, nausea and vomiting were prominent complaints and significant suppression of WBC, platelets, and reticulocytes were seen in both patients with maximum depression ten to 15 days after beginning treatment. Marrow function recovered within three weeks of the initial dose. When 0.2 mg/kg/d of 5-aza was given orally with THU (regimen V, Table 1) F reticulocyte levels comparable to those achieved with the optimal SC regimen (regimen III of 2 mg/kg/d) were observed with no evidence of cytotoxicity.

An oral THU dose of 1.5 to 2 mg/kg (divided in two doses, the first given one hour before, the second with 5-aza; fasting) resulted within 24 hours in a suppression of peripheral blood WBC cytidine deaminase levels to 31% and 33% of pretherapy levels in patients A and C, respectively. Cytidine deaminase levels had returned to 85% to 100% of pretherapy values seven days later when the drug was given for two consecutive days each week. When given three consecutive days each week (see regimen V), day 7 cytidine deaminase

Table 2. Hematologic Response to Various Doses of 5-Azacytidine for Patients A and C

Patient	Dose*	Days of Therapy	Hb(g/dL)	Reticulocytes (%)	HbF (%)	F Cells (%)
A	Pre		8.5 ± 1.0†	12.0 ± 4.0 (4.3)	1.5 ± 0.7	9.3 ± 1.7
	I	125–160	11.3 ± 0.8	15.3 ± 9.1 (4.5)	7.6 ± 1.5	41.4 ± 5.7
	II	165–200	11.9 ± 0.6	16.8 ± 6.4 (5.0)	7.1 ± 0.2	35.2 ± 3.5
	III	203–240	12.0 ± 0.7	17.1 ± 5.2 (5.1)	8.0 ± 0.8	32.6 ± 2.8
	IV	245–280	11.7 ± 0.3	17.1 ± 7.6 (5.6)	8.6 ± 1.0	36.2 ± 5.8
	V	415–464	11.3 ± 0.7	12.5 ± 4.1 (3.5)	9.6 ± 0.7	43.0 ± 2.7
C	Pre		8.0 ± 0.5†	13.9 ± 2.8 (3.9)	2.9 ± 0.1	14.4 ± 1.2
	V	140–180	9.2 ± 0.3	15.3 ± 5.5 (3.4)	17.5 ± 0.6	63.4 ± 3.9

Parentheses indicate the mean absolute reticulocyte level × $10^5/\mu L$.

*See Table 1 for doses of 5-aza.

†Patient A had 46% HbA, and patient C had 56% HbA due to previous transfusions.

levels were 54% and 69% of pretherapy levels for both patients.

Effect of various 5-aza doses on other hematologic measurements: hemoglobin, reticulocyte count, percentage of HbF and percentage of F cells. Table 2 summarizes average hemoglobin concentrations, reticulocyte counts, percentage of HbF, and percentage of F cells obtained on patients A and C on various regiments of 5-aza. Only those data obtained when no transfused cells were present are summarized. Drug regimens were changed before hemoglobin concentrations had returned to pretreatment levels, but after F reticulocyte levels had dropped to pretreatment levels.

Hemoglobin concentrations rose in both patients A and C while they were on 5-aza (Table 2). Patient A's levels were consistently higher than those of Patient C even though HbF levels behaved conversely. Although patient B's hemoglobin values rose on regimen III from 8.5 g/dL (pretherapy) to 11.5 g/dL (day 84), he was too noncompliant to obtain sufficient data after transfused cells had disappeared. Patient D left the study (see Materials and Methods) shortly after a second dose of 5-aza. Except for transient suppression of reticulocytes following the inception of high-dose oral 5-aza therapy (2 mg/kg/d + 200 mg THU), the proportions of reticulocytes in patients A and C were unchanged during treatment.

Because we were unwilling to allow our patients to return to their pretreatment hemoglobin concentrations before changing dosage regimens, we cannot conclude from these data whether one drug regimen results in a higher hemoglobin concentration than another regimen. It is apparent, however, that hemoglobin concentrations remained persistently higher than pretreatment levels in patient A over a prolonged period of time (500 days posttherapy).

HbF/F cell. During the first 300 days of SC therapy in patient A, negligible differences in the amount of HbF/F cell were seen (Table 3) even though F reticulocyte production and F cell levels increased dramatically (Table 2). In contrast, during oral 5-aza therapy, the amount of HbF/F cell increased 37% in patient A (from 7.6 to 10.4 pg) and 103% in patient C (from 5.5 to 11.2 pg) (Table 3). No changes in HbF/F cell were seen in the short time that patients B and D were followed.

RBC indices and "dense cells." After 100 days of therapy both patients A and C demonstrated increases in their MCV and MCH (Table 3). In patient A, the mean MCV and MCH were increased the most during regimen III (114 ± 2 Fl and 40 ± 1 pg) and regimen V (116 ± 4 Fl and 40 ± 1 pg). Both patient A and C had normal serum B_{12} and red cell folate levels throughout therapy. The MCHC as measured by an electronic cell counter (patients A and C) or by the pthalate ester technique (patient C only) did not change (Table 3). The proportion of erythrocytes with a MCHC >37 g/dL was followed weekly in patient C (Fig 2). Dense cells disappeared between day 40 and 90, coincident with a decrease in reticulocytes which occurred during the period of marrow depression and gastrointestinal toxicity following a toxic high oral dose of 5-aza (2 mg/kg/d) given with THU. Patient A (data not shown) showed a similar suppression of dense cells and reticulocyte counts at this dosage schedule. After adjustment of dosage (Table 1, oral regimen V) when F reticulocyte levels remained above 20% and F cell levels ranged from 65% to 83%, the percentage of dense cells varied between 4% and 8%. Pretreatment levels were similar (4% to 6%). However, the patient had been transfused prior to drug therapy and had 46% normal red cells in his blood at the beginning of observation. No correlation between the proportion of dense cells and vasoocclusive crisis (as noted by Fabry et al[24]) was observed in patient C. However, only two assays were performed on patient C during that period of therapy when crises occurred (day 0 through 20).

Markers of "fetal erythropoiesis." Because all patients

Table 3. HbF Levels and Erythrocyte Indices on Patients A and C

Patient	Day of Therapy	HbF (%)	F Cells (%)	HbF/F Cell (pg)	MCV (fl)	MCH (pg)	MCHC* (g/dL)
A	0	1.8	8	7.6	95	34	35.8
	101	6.5	33	7.2	105	37	35.2
	200	7.3	40	6.9	108	38	35.2
	301	6.8	33	8.0	110	38	34.5
	410	8.6	42	8.1	121	40	33.7
	500	9.5	40	10.4	125	44	35.2
C	0	2.9	18	5.5	98	34	34.7
	52	10.9	50	7.4	101	34	33.7
	100	15.1	64	9.9	124	42	33.9
	146	16.8	62	11.3	126	42	33.3
	203	16.6	62	11.2	123	42	34.2

*Values on patient A were determined by Coulter Counter (Hialeah, Fla) and on patient C by phthalate ester technique.

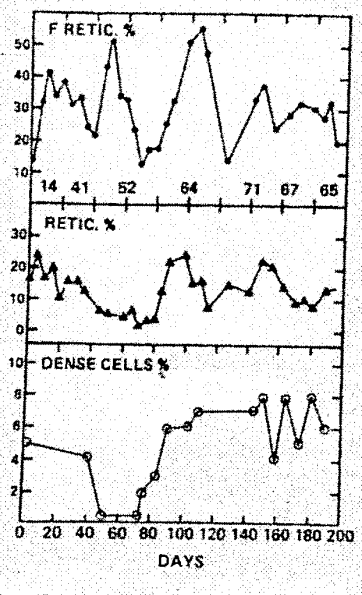

Fig 2. Alterations in F reticulocytes, total reticulocytes, and the proportion of dense cells (MCHC <37 g/dL) in patient C. Note that suppression in total reticulocytes followed administration of oral 5-azacytidine (2 mg/kg/d for three days) with THU begun on day 35. The numbers in the top panel refer to the percentage of F cells present on the corresponding days. Regimen III was given days 0 through 35, and regimen V was given on days 140 through 200.

had been transfused before starting 5-aza treatment, comparison of erythrocyte antigens and enzymes before and during therapy would be meaningless. Markers of the fetal erythropoiesis were examined when maximal levels of HbF (8% to 10% in patient A; 18% to 20% in patient C) were attained. The activity of galactokinase, an erythrocyte enzyme which is elevated prenatally,[18] was within adult levels.

The percentage of Gγ globin chains as a fraction of total HbF was 53% in patient A and 73% in patient C (normal newborn level, 70%; normal adult level, <50%). Gilman and Huisman[25] have recently shown that two *Hind*III–*Hin*cII restriction enzyme polymorphism haplotypes around the Gγ-Aγ-β globin genes are associated with either high or low Gγ levels. According to their analysis, patient A, homozygous for haplotype + − − −, should have a low Gγ level (<48%). Patient C, homozygous for haplotype + − + + would be predicted to have a high (>56%) Gγ level. The red cell i-antigen titer was elevated in patient A (1/64 titer) and undetectable in patient C (normal adult titer 0) cord blood titer (1/128). Using chromatographic methods,[26] Schroeder could detect no embryonic hemoglobin (Gower 1 or Gower 2) in either patient using methodology which would detect as little as 0.03%. α-Fetoprotein and carcinoembryonic antigen levels remained normal during therapy.

Vasoocclusive crisis. Vasoocclusive crisis did not decrease in number in the first 130 days of therapy in patient A (see previous report[5]). However, with continued parenteral treatment crisis frequency decreased to 10/175 days (Table 4). During oral therapy the number of crisis days was also

Table 4. Frequency of Vaso-occlusive Painful Crisis in Patients A and C Treated With Subcutaneous (SC) and Oral (PO) 5-Azacytidine

	A		C	
	Day	Crisis*	Day	Crisis*
Pretreatment	−166–0	70	−200–0	100
Initial trial	0–125†	70	—	—
SC 5-Aza	126–300	10	0–35	20
PO 5-Aza	301–500	15	36–200	0

*Days in crisis as defined in Materials and Methods.
†Previous report[5]; monthly or every-two-week pulses of IV or SC 5-aza.

decreased (15 of 200 days). Patient C responded much more dramatically. After day 30 of treatment, he had no clearcut further painful crisis. It is important to note that these observations were uncontrolled, and that the patients knew when they were receiving treatment and when the dosages were altered. Furthermore, each patient received intense emotional and psychiatric support from medical and nursing personnel. For these reasons, no conclusions can be drawn concerning amelioration of the clinical features of the disease during 5-aza therapy.

DISCUSSION

Mechanism of action. All four SS patients treated with 5-aza demonstrated significant increases in HbF production. The mechanism of the increase is unknown, but two major hypotheses have been advanced. In one, late erythroid precursors are "reprogrammed" through some action of 5-aza[27]; in the other, early erythroid precursors, with an inherent program for increased production of HbF, are "recruited" concomitant with the cytotoxic destruction of later precursors.[3] Some light is shed on the question by examining the timing of the in vivo F reticulocyte response to the drug. Three of our four patients exhibited a rapid response (Fig 1) and *no* decline in total reticulocyte production. Only patient D failed to show a significant immediate rise in F reticulocytes. Variation in the rapidity of the F reticulocyte response was also observed in six SS patients treated with a single course of 5-aza at the National Institutes of Health by Ley and Nienhuis and assayed in our laboratory: five of them responded rapidly, but one did not.[27] The rapid increase in F − reticulocytes within 24 to 48 hours of treatment without suppression of total reticulocytes suggests that, in most patients, 5-aza reprograms late erythroid precursors to produce HbF. Patient D exhibited gastrointestinal and bone marrow toxicity after the single course of treatment which produced the delayed response. The one patient with a delayed response treated at the National Institutes of Health behaved in similar fashion. It is conceivable that the late response in these two patients reflects the cytotoxic "recruitment" phenomenon, but that responses to doses of 5-aza not accompanied by toxicity reflect the alternate "reprogramming" effect.

It is intriguing to note that absolute reticulocyte levels in our patients were not significantly different from pretherapy levels ($P > .10$) on any of the drug regimens described (see

Table 2). Furthermore, at no time in patients A and C did transient declines in total reticulocyte production precede elevations in F reticulocyte levels nor were reticulocyte counts lower during regimens that produced higher F reticulocyte responses. These observations suggest that increased F cell production was not associated with or preceded by a decline in erythroid activity and further makes recruitment of early precursors, as a result of marrow suppression, an unlikely cause of increased HbF production.

Improvement in anemia. With sustained increased F reticulocyte production, hemoglobin concentration rose, as did the MCV, MCH, and the amount of HbF/F cell. The MCHC, however, remained constant. There was also a suggestion that clinical symptoms (painful crises) decreased, raising questions of the relationships between these effects.

The concentration of HbS within the red cell is a major determinant of HbS polymerization.[28] A previous report[6] suggested that improvement in anemia seen in 5-aza treatment is associated with decreased MCHC and the disappearance from the blood of very dense cells (ie, those with the higher MCHC). However, in those patients, the disappearance of dense cells and the change in MCHC were also associated with transient reticulocyte suppression.[6] In our patients A and C, dense cells decreased only when reticulocytes were suppressed after the patients were given ten times the optimal oral dose of 5-aza/THU. Recent data by Noguchi et al[17] suggest that these dense cells are relatively young cells and therefore might be expected to disappear if reticulocyte levels fall. However, during optimal therapy (regimens III and V), when reticulocyte percentages were *not* decreased, Hb levels remained elevated, the MCHC did not change, and dense cells persisted (ie, new ones were formed as fast as old ones were removed). It appears, therefore, that the partial compensation for the anemia in these patients is not attributable to either a decreased MCHC or the disappearance of dense cells.

A second possible explanation for the decreased anemia in our patients is their increased HbF production. Initially, improvement in anemia was associated with increased F cell production without an increase in HbF/F cell. This indicates that a decrease in anemia in SS patients may be accomplished by increasing F cell production without increasing HbF/F cell. In contrast, HbF/F cell did increase on oral regimens. Surprisingly, in both patients A and C the increase in the MCH (10 pg and 8 pg, respectively) was greater than the increase in HbF/F cell (3.8 pg in A and 4.7 pg in C) indicating that HbS/cell also had increased. Assuming that the MCH of F cells and non-F cells are equal, the percentage of total hemoglobin per cell attributed to HbF did not increase substantially in patient A (22% to 24%, day 0 to 500), but did rise in patient C (16% to 27%, day 0 to day 200). Despite these differences in HbF production, *both* patients had less anemia; the lower HbF level in patient A being associated with *higher* average hemoglobin levels. It appears that increased HbF production alone cannot account for the changes in anemia seen in our patients.

Fetal characteristics of RBCs. Additional fetal erythrocyte characteristics were observed during the course of therapy. Elevated MCV was seen in both patients. Erythrocyte i-antigen titers were increased in patient A and not in patient C even though patient C had the higher F cell level at the time of assay. Only patient A had higher $G\gamma/A\gamma$ ratios than predicted from their restriction enzyme polymorphism haplotypes.[25] Fetal levels of erythrocyte enzymes were not observed in either patient. This mixed pattern of fetal and adult erythrocyte markers is more consistent with the changes seen with stress erythropoiesis[19,29,30] rather than a true pattern of fetal erythropoiesis.

Variability of response. We have observed that the rapidity of response to 5-aza varies between patients. Although the reasons for this variability are unknown, cytotoxic effects clearly are associated with the delayed F reticulocyte response. Because baseline levels of F cell production in SS disease are genetically determined[7] and since pretherapy levels of F reticulocytes were predictive of subsequent F reticulocyte responses (Table 1), it might be expected that SS patients with high baseline F reticulocyte levels may need less intensive therapy than patients with low (<5%) F reticulocyte levels. However, it is unclear what minimal percentage of F reticulocyte, if any, can reduce the clinical severity of sickle cell disease. Each person brings to therapy his own genetic constraints on F cell production. Such constraints may influence the amount of drug and the magnitude of response needed to reduce clinical symptoms. Furthermore, it is not clear whether improvements in any one hematologic parameter (percentage of HbF, F reticulocyte level, HbF/F cell, Hb level, and percentage of dense cells) predicts clinical improvement as defined by frequency of severity of vasoocclusive crisis. The absence of such a definable marker makes assessment of therapeutic manuevers more difficult.

Overall role of 5-aza in SS disease. We have increased HbF in a limited number of SS patients. Little toxicity was observed, but the long-term effects of this form of therapy are unknown. Although probably carcinogenic in animals,[10] little information is available regarding the carcinogenic potential of 5-aza in humans. In the absence of controlled clinical trials, we cannot determine whether elevations in HbF levels, produced as described here, will significantly alter the clinical course of patients with sickle cell disease. The question of carcinogenicity impedes such trials, but other cell-cycle–specific agents (which may be less carcinogenic) have been shown to increase HbF in animals[31,32] and in humans.[33] These agents should be evaluated in a manner similar to that outlined in this report before controlled clinical trials are begun.

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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

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Summary

Background Drug treatments for patients with high-risk myelodysplastic syndromes provide no survival advantage. In this trial, we aimed to assess the effect of azacitidine on overall survival compared with the three commonest conventional care regimens.

Methods In a phase III, international, multicentre, controlled, parallel-group, open-label trial, patients with higher-risk myelodysplastic syndromes were randomly assigned one-to-one to receive azacitidine (75 mg/m² per day for 7 days every 28 days) or conventional care (best supportive care, low-dose cytarabine, or intensive chemotherapy as selected by investigators before randomisation). Patients were stratified by French–American–British and international prognostic scoring system classifications; randomisation was done with a block size of four. The primary endpoint was overall survival. Efficacy analyses were by intention to treat for all patients assigned to receive treatment. This study is registered with ClinicalTrials.gov, number NCT00071799.

Findings Between Feb 13, 2004, and Aug 7, 2006, 358 patients were randomly assigned to receive azacitidine (n=179) or conventional care regimens (n=179). Four patients in the azacitidine and 14 in the conventional care groups received no study drugs but were included in the intention-to-treat efficacy analysis. After a median follow-up of 21·1 months (IQR 15·1–26·9), median overall survival was 24·5 months (9·9–not reached) for the azacitidine group versus 15·0 months (5·6–24·1) for the conventional care group (hazard ratio 0·58; 95% CI 0·43–0·77; stratified log-rank p=0·0001). At last follow-up, 82 patients in the azacitidine group had died compared with 113 in the conventional care group. At 2 years, on the basis of Kaplan-Meier estimates, 50·8% (95% CI 42·1–58·8) of patients in the azacitidine group were alive compared with 26·2% (18·7–34·3) in the conventional care group (p<0·0001). Peripheral cytopenias were the most common grade 3–4 adverse events for all treatments.

Interpretation Treatment with azacitidine increases overall survival in patients with higher-risk myelodysplastic syndromes relative to conventional care.

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Introduction

Myelodysplastic syndromes are malignant diseases of bone-marrow stem-cells, characterised by ineffective haemopoiesis leading to peripheral-blood cytopenias and, in many patients, progression to acute myeloid leukaemia.[1,2] Myelodysplastic syndromes are categorised morphologically with the French–American–British (FAB) and, more recently, WHO[3,4] classifications. Individual prognosis is determined using the international prognostic scoring system.[5]

Patients with myelodysplastic syndromes who had intermediate-2 or high-risk scores on the international prognosis scoring system (known as higher-risk myelodysplastic syndromes) have a median survival of 1·2 years or 0·4 years, respectively,[5] and a high-risk for progression to acute myeloid leukaemia.[5] Although increasing survival and suppression of leukaemic transformation are the primary goals of treatment,[6] no treatment strategies other than allogeneic stem-cell transplantation offer meaningful potential to change the natural history of the disease.[7–15] Results of a Cancer and Leukemia Group B (CALGB) trial comparing treatment with azacitidine, a DNA methyltransferase inhibitor, with best supportive care suggested improved overall survival with azacitidine, but the study was inconclusive because of its crossover design and absence of an active comparator.[16]

This large, prospective, randomised, phase III, clinical trial was done to assess the effect of treatment on overall survival with azacitidine. The control arm included the three most commonly used treatments in higher-risk myelodysplastic syndromes (best supportive care, low-dose cytarabine, or intensive chemotherapy).[6–15,17]

Methods

Patients

Patients were eligible for enrolment if they were aged 18 years or older, with higher-risk myelodysplastic syndromes (an international prognosis scoring system

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rating of intermediate-2 or high risk) and FAB-defined refractory anaemia with excess blasts, refractory anaemia with excess blasts in transformation, or chronic myelomonocytic leukaemia[3] with at least 10% bone-marrow blasts and a white-blood-cell count lower than 13×10^9 cells per L. Patients needed an Eastern Cooperative Oncology Group (ECOG) performance status of 0–2 and an estimated life expectancy of at least 3 months. Patients with therapy-related myelodysplastic syndrome, previous azacitidine treatment, or planned allogeneic stem-cell transplantation were excluded.

This phase III, international, multicentre, randomised, controlled, parallel-group, open-label trial was done 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. Enrolment to the trial and monitoring were done by site investigators and central pathology reviewers with standardised central review of all cytogenetic data.

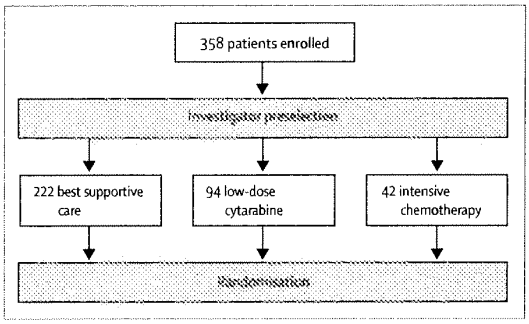

Figure 1: **Investigator preselection**
Before randomisation, investigators preselected the most appropriate of the three conventional care regimens for all patients on the basis of age, general condition, comorbidities, and patient preference. Patients randomised to conventional care were to receive the investigator preselected treatment option.

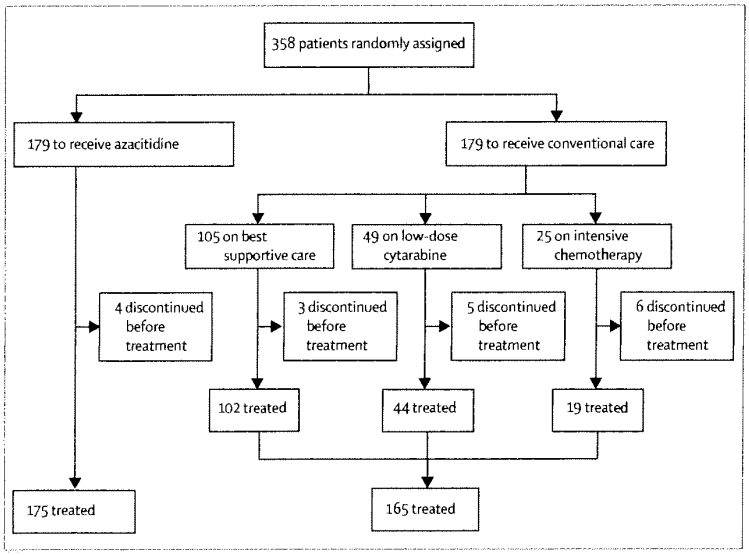

Figure 2: **Trial profile**

Study design

Before randomisation, investigators determined which of the three conventional care treatments (best supportive care, low-dose cytarabine, or intensive chemotherapy) was most appropriate for each patient, with clinical judgment on the basis of age, ECOG performance status, and comorbidities (figure 1). Patients were then randomly assigned one-to-one to receive azacitidine or conventional care regimens (figure 2). No crossover was allowed, and use of erythropoietin or darbepoetin was prohibited.

Patients were stratified by investigators according to FAB and international prognostic scoring system classifications.[3,5] Randomisation was done centrally, with allocation by telephone; patients were assigned to treatment in blocks of four within each stratum. The randomisation sequence was computer generated independently by Pharmaceutical Product Development (Wilmington, NC, USA). An independent data-safety monitoring board reviewed safety data and did an unblinded review of a scheduled interim efficacy analysis.

During the treatment phase of the trial, all treatments were continued until study completion (12 months after the last patient was assigned) or discontinuation due to relapse, unacceptable toxicity, or disease progression defined by the International Working Group (IWG 2000) criteria for myelodysplastic syndromes.[18] Azacitidine was given subcutaneously at 75 mg/m² per day for 7 days every 28 days (delayed as needed until blood-count recovery), for at least six cycles. Conventional care regimens were given as follows: best supportive care only (including blood product transfusions and antibiotics with granulocyte-colony-stimulating factor for neutropenic infection); low-dose cytarabine, 20 mg/m² per day subcutaneously for 14 days, every 28 days (delayed as needed until blood-count recovery) for at least four cycles; or intensive chemotherapy (induction with cytarabine 100–200 mg/m² per day by continuous intravenous infusion for 7 days, plus 3 days of either intravenous daunorubicin [45–60 mg/m² per day], idarubicin [9–12 mg/m² per day], or mitoxantrone [8–12 mg/m² per day]). Patients who achieved complete or partial remission after induction (defined by the International Working Group criteria for acute myeloid leukaemia[19]) received one or two consolidation courses with reduced doses of the cytotoxic drugs used for induction followed by best supportive care. All patients could receive best supportive care as needed. After treatment discontinuation, all patients were followed up until death or study completion.

Assessment of efficacy and safety

Efficacy analyses were by intention to treat. Safety analyses included all patients who received at least one dose of study drug and one or more safety assessments thereafter. The primary endpoint was overall survival,

analysed by comparison of the azacitidine and combined conventional care groups. A supportive analysis of overall survival assessed the potential effects of predefined subgroups on the basis of age; FAB subtype, risk group (intermediate-2 or high), and cytopenias (grades 0 or 1 and 2 or 3); cytogenetics (good, intermediate, and poor); –7/del(7q) cytogenetic abnormality, WHO classification; and serum lactate dehydrogenase. Another supportive analysis of overall survival, on the basis of investigator preselection, compared the azacitidine subgroups with the individual treatments of conventional care.

Secondary efficacy endpoints were time to transformation to acute myeloid leukaemia, haematological response, and improvement assessed with IWG 2000 criteria for myelodysplastic syndromes,[18] independence from red-blood-cell transfusions for 56 consecutive days or more, number of infections requiring intravenous antimicrobials, and occurrence of adverse events. Bone-

	Total ITT		BSC only (n=222)		Low-dose cytarabine (n=94)		Intensive chemotherapy (n=42)	
	Azacitidine (n=179)	CCR (n=179)	Azacitidine (n=117)	BSC (n=105)	Azacitidine (n=45)	Low-dose cytarabine (n=49)	Azacitidine (n=17)	Intensive chemotherapy (n=25)
Age (years)	69 (42–83)	70 (38–88)	69 (52–83)	70 (50–88)	69 (42–82)	71 (56–85)	63 (45–78)	65 (38–76)
≤64	57 (32%)	43 (24%)	33 (28%)	24 (23%)	14 (31%)	7 (14%)	10 (59%)	12 (48%)
≥65	122 (68%)	136 (76%)	84 (72%)	81 (77%)	31 (69%)	42 (86%)	7 (41%)	13 (52%)
Sex								
Men	132 (74%)	119 (67%)	81 (69%)	67 (64%)	39 (87%)	35 (71%)	12 (71%)	17 (68%)
Women	47 (26%)	60 (34%)	36 (31%)	38 (36%)	6 (13%)	14 (29%)	5 (29%)	8 (32%)
FAB classification								
RAEB	104 (58%)	103 (58%)	69 (59%)	68 (65%)	27 (60%)	25 (51%)	8 (47%)	10 (40%)
RAEB-T	61 (34%)	62 (35%)	38 (33%)	30 (29%)	15 (33%)	19 (39%)	8 (47%)	13 (52%)
CMMoL	6 (3%)	5 (3%)	5 (4%)	4 (4%)	1 (2%)	1 (2%)	0	0
AML	1 (1%)	1 (1%)	0	0	1 (2%)	0	0	1 (4%)
IPSS classification								
Intermediate-1	5 (3%)	13 (7%)	4 (3%)	9 (9%)	1 (2%)	2 (4%)	0	2 (8%)
Intermediate-2	76 (43%)	70 (39%)	48 (41%)	46 (44%)	22 (49%)	21 (43%)	6 (35%)	3 (12%)
High	82 (46%)	85 (48%)	57 (49%)	46 (44%)	19 (42%)	21 (43%)	6 (35%)	18 (72%)
Karyotype risk								
Good	83 (46%)	84 (47%)	53 (45%)	47 (45%)	24 (53%)	28 (57%)	6 (35%)	9 (36%)
Intermediate	37 (21%)	39 (22%)	25 (21%)	23 (22%)	7 (16%)	12 (25%)	5 (29%)	4 (16%)
Poor	50 (28%)	50 (28%)	33 (28%)	31 (30%)	13 (29%)	8 (16%)	4 (24%)	11 (44%)
Missing	9 (5%)	6 (3%)	6 (5%)	4 (4%)	1 (2%)	1 (2%)	2 (12%)	1 (4%)
WHO classification								
RAEB-1	14 (8%)	17 (10%)	8 (7%)	13 (12%)	3 (7%)	3 (6%)	3 (18%)	1 (4%)
RAEB-2	98 (55%)	95 (53%)	63 (54%)	60 (57%)	27 (60%)	24 (49%)	8 (47%)	11 (44%)
CMMoL-1	1 (1%)	0	1 (1%)	0	0	0	0	0
CMMoL-2	10 (6%)	5 (3%)	8 (7%)	3 (3%)	1 (2%)	0	1 (6%)	2 (8%)
AML	55 (31%)	58 (32%)	36 (31%)	27 (26%)	14 (31%)	20 (41%)	5 (29%)	11 (44%)
Indeterminate	1 (1%)	4 (2%)	1 (1%)	2 (2%)	0	2 (4%)	0	0
ECOG performance status								
0	78 (44%)	80 (45%)	47 (40%)	36 (34%)	21 (47%)	29 (59%)	10 (59%)	15 (60%)
1	86 (48%)	86 (48%)	59 (50%)	59 (56%)	21 (47%)	17 (35%)	6 (35%)	10 (40%)
2	13 (7%)	10 (6%)	11 (9%)	8 (8%)	1 (2%)	2 (4%)	1 (6%)	0
Missing	2 (1%)	3 (2%)	0	2 (2%)	2 (4%)	1 (2%)	0	0
Time since original diagnosis (years)								
<1	92 (51%)	95 (53%)	53 (45%)	53 (51%)	29 (64%)	28 (57%)	10 (59%)	14 (56%)
1–2	37 (21%)	45 (25%)	29 (25%)	27 (26%)	7 (16%)	12 (25%)	1 (6%)	6 (24%)
2–3	20 (11%)	10 (6%)	14 (12%)	6 (6%)	4 (9%)	3 (6%)	2 (12%)	1 (4%)
≥3	30 (17%)	29 (16%)	21 (18%)	19 (18%)	5 (11%)	6 (12%)	4 (24%)	4 (16%)

Data are median (range) or number (%). CCR=conventional care regimen. ITT=intention to treat. BSC=best supportive care. FAB=French–American–British. RAEB=refractory anaemia with excess blasts. RAEB-T=RAEB in transformation. CMMoL=chronic myelomonocytic leukaemia. AML=acute myeloid leukaemia. IPSS=International prognostic scoring system. ECOG=Eastern Cooperative Oncology Group.

Table 1: **Baseline demographics and disease characteristics by treatment group and investigator preselection**

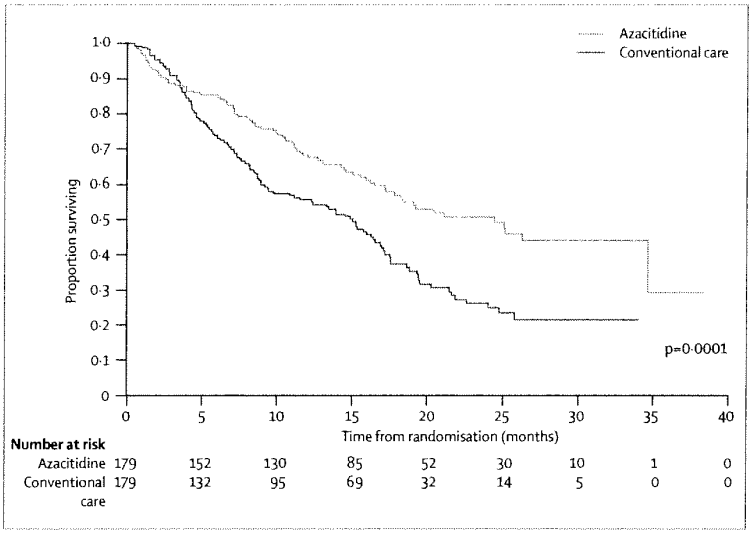

Figure 3: **Overall survival**

marrow samples were collected every 16 weeks during active treatment and as clinically indicated during follow-up. Infections requiring intravenous antimicrobials were counted from randomisation to last study visit. Adverse events were assessed with the National Cancer Institute's Common Toxicity Criteria, version 2.0.

Statistical methods

This study was designed with 90% power—on the basis of a log-rank analysis—to detect an HR of 0·60 for overall survival in the azacitidine group compared with that in the conventional care group, with a two-sided α of 0·05. The protocol specified that about 354 patients were to be randomly assigned 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 and a minimum follow-up of at least 12 months for all patients, however, necessitated a longer study period. With a study period of 42 months and 195 deaths, the study had 95% power under the assumptions of the design. One interim analysis was done with an O'Brien-Fleming monitoring boundary with a Lan-DeMets α spending function to control the overall α at 0·05 (data not shown).[20]

Overall survival was defined as the time from randomisation to death from any cause. Patients who remained alive were censored at the time of last follow-up. Time to transformation to acute myeloid leukaemia was measured from randomisation to development of 30% or greater bone-marrow blasts. Patients free from acute myeloid leukaemia transformation were censored for this analysis at the time of last adequate bone-marrow sample. Randomisation and analyses were stratified on FAB subtype and international prognostic scoring system group. Time-to-event curves were estimated with the Kaplan-Meier method[21] and compared with stratified log-rank tests (primary analysis). Stratified Cox proportional hazards regression models[22] were used to estimate hazard ratios (HRs) and associated 95% CIs. The primary analysis of overall survival between the azacitidine and combined conventional care groups used the stratified Cox proportional hazards model without any covariate adjustments to estimate the HR. Cox proportional hazards regression with stepwise selection was used to assess the baseline variables of sex, age, time since original diagnosis of myelodysplastic syndrome, ECOG performance status, number of previous red-blood-cell transfusions, number of previous platelet transfusions, measurements of haemoglobin, platelets, absolute neutrophil count, and lactate dehydrogenase, bone-marrow blast percentage, and presence or absence of cytogenetic –7/del(7q) abnormality. The final model included ECOG performance status, lactate dehydrogenase, haemoglobin, number of previous red-blood-cell transfusions, and presence or absence of the cytogenetic –7/del(7q) abnormality. Supportive overall survival analyses used the final Cox proportional hazards model. The consistency of treatment effect across subgroups was assessed with the difference in likelihood ratio between the full model with treatment subgroup and treatment-by-subgroup interaction, and the reduced model without the interaction. Additional supportive efficacy analyses by investigator preselection compared the azacitidine subgroups with the individual treatments that comprised conventional care (figure 1).

Haematological response, transfusion independence, and haematological improvement in the azacitidine and conventional care groups were compared with Fisher's exact test. The rate of infection requiring intravenous antimicrobials was calculated with the number of recorded infections treated with intravenous antimicrobials divided by the total number of patient-years of follow-up. The relative risk of infection was calculated as the rate of infection in patients taking azacitidine compared with the rate in those receiving conventional care. The Mantel-Haenszel estimate of the common relative risk, the associated 95% CI, and the test that it equals unity were calculated.[23] Analyses were done with SAS (version 9.13).

This study is registered with ClinicalTrials.gov, number NCT00071799.

Role of the funding source

The principal investigator and leading coinvestigators designed and did the study, provided oversight for the analysis of the data by Celgene, and wrote the article in consultation with Celgene. Additionally, Celgene elicited independent review of the statistical analysis plan by Kenneth J Kopecky (Fred Hutchinson Cancer Research Center, SW Oncology Group Statistical Center, Seattle, WA, USA) and Gary G Koch (University of North Carolina at Chapel Hill, Director, Biometric Consulting Lab, Chapel Hill, NC, USA) and independent review of the analyses, interpretation of the results, and review of this paper by

Gary G Koch. The corresponding author and coauthors had access to all the trial data and had final responsibility for the decision to submit for publication.

Results

Between Feb 13, 2004, and Aug 7, 2006, 358 patients (intention-to-treat population) at 79 sites from 15 countries were randomly assigned to receive either azacitidine (n=179) or conventional care regimens (n=179). Of those assigned to conventional care, 105 were to receive best supportive care, 49 low dose cytarabine, and 25 intensive chemotherapy (figure 2). Median age was 69 years (range 38–88 years) with 258 (72%) of 358 patients age 65 years or older. Baseline characteristics were well balanced between the azacitidine and conventional care groups (table 1). The investigator preselection subgroups showed some imbalances as expected: namely, patients selected to receive intensive chemotherapy were younger and had better ECOG performance status and higher-risk disease (table 1). According to the WHO classification, 113 patients (32%) fulfilled criteria for acute myeloid leukaemia (marrow-blast percentage 20% or greater). The following protocol deviations were reported: 18 patients with international prognosis scoring system score of intermediate-1 were enrolled after central review (five in the azacitidine group; 13 in the conventional care group) and investigators decided to give eight patients (four in the azacitidine group and four in the conventional care group [two best supportive care; one low-dose cytarabine, and one intensive chemotherapy]) allogeneic transplantation during follow-up. Four patients in the azacitidine group and 14 in the conventional care group never received study drug but were followed for overall survival and are included in the intention to treat analysis (figure 2). All patients with protocol deviations are included in the intention to treat analysis.

Azacitidine was given for a median of nine cycles (IQR four to 15), and 151 (86%) of 175 of patients who received azacitidine remained on 75 mg/m² per day throughout the study with no dose adjustments. The median azacitidine cycle-length was 28 days (IQR 28–35); 862 (54%) of the 1611 cycle-lengths were 28 days, 413 (26%) 29–35 days, and 336 (21%) longer than 35 days. Low-dose cytarabine was given for a median of four and a half cycles (IQR two to eight); 59 (29%) of 201 cycle-lengths were 28 days, 82 (41%) 29–35 days, and 60 (30%) longer than 35 days, the overall median was 35 days (IQR 28–36). Intensive chemotherapy was given for a median of one cycle (IQR one to three), and best supportive care for a median 6·2 months (IQR 3·6–10·3).

At the time of last follow-up, 82 patients in the azacitidine group had died compared with 113 in the conventional care group. After a median follow-up of 21·1 months (IQR 15·1–26·9), median Kaplan-Meier overall survival was 24·5 months (9·9–not reached) in the azacitidine group compared with 15 months (5·6–24·1) in the conventional care group, a difference of 9·4 months (stratified log-rank p=0·0001; figure 3). The HR for overall survival was 0·58 (95% CI 0·43–0·77). Kaplan-Meier survival curves for the azacitidine and conventional care groups separated permanently after about 3 months, at which time 140 (78%) of 179 patients receiving azacitidine had completed three cycles of

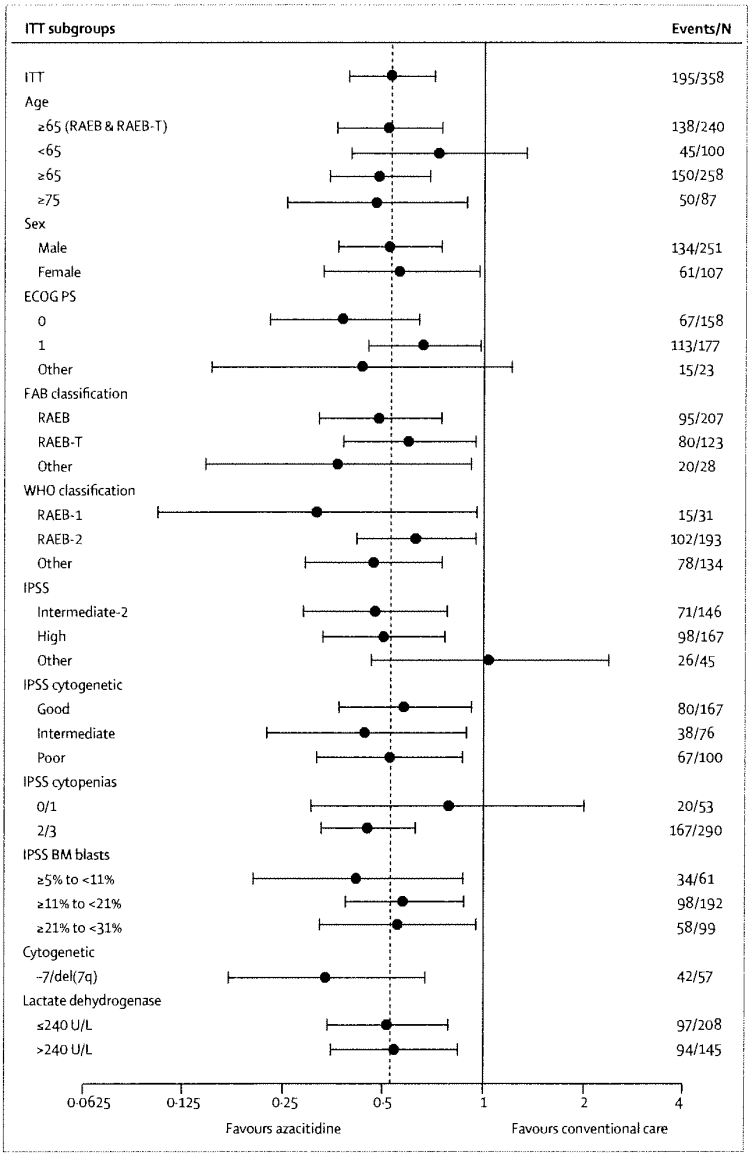

Figure 4: **Hazard ratio and 95% CI for overall survival in the intention-to-treat analysis**
Hazard ratios and CIs determined with stratified Cox proportional hazards model adjusted for treatment, subgroup, Eastern Cooperative Oncology Group performance status (ECOG PS), lactate dehydrogenase, haemoglobin, number of previous red-blood-cell transfusions, and presence or absence of the cytogenetic -7/del(7q) abnormality. No subgroup-by-treatment interactions were significant (p>0·20). The horizontal axis uses a logarithmic scale. The dotted line is the hazard ratio in the primary intention to treat (ITT) analysis; the hazard ratio and CI are from the stratified Cox regression model with treatment as the only term. FAB=French-American-British. RAEB=refractory anaemia with excess blasts. RAEB-T=RAEB in transformation. IPSS=international prognostic scoring system. BM=bone marrow.

treatment (figure 3). At 2 years, on the basis of Kaplan-Meier estimates, 50·8% (95% CI 42·1–58·8) of patients in the azacitidine group were alive compared with 26·2% (18·7–34·3) in the conventional care group (p<0·0001).

The supportive analysis of all predefined subgroups of patients showed consistency of the azacitidine effect on overall survival compared with conventional care (figure 4). In particular, overall survival was better for azacitidine than conventional care in all the cytogenetic subgroups on the international prognosis scoring system (poor prognosis HR 0·53, 95% CI 0·32–0·87, p=0·012; intermediate prognosis 0·44, 0·22–0·88, p=0·021; and good prognosis 0·59, 0·37–0·92, p=0·021). In patients with –7/del(7q), median Kaplan-Meier overall survival was 13·1 months (IQR 3·9–24·5; 95% CI 9·9–24·5) in the azacitidine group (n=30) compared with 4·6 months (2·9–9·3; 3·5–6·7) in the conventional care group (n=27) giving an HR of 0·34 (95% CI 0·17–0·67, p=0·0017; figure 4). Additionally, sensitivity analyses exploring the effect of the eight patients who received allogeneic stem-cell transplantation included in the intention-to-treat analyses, determined they did not affect the significance of the overall survival results (data not shown).

Similar to the primary overall survival comparison (azacitidine *vs* conventional care), results from the investigator preselection subgroup analysis of overall survival showed significant differences favouring the study drug between azacitidine and best supportive care (9·6 months, p=0·0045) and azacitidine and cytarabine (9·2 months, p=0·0006). The difference in the comparison between azacitidine (n=17) and intensive chemotherapy (n=25), however, was not significant (9·3 months, p=0·51, table 2).

Median time to acute myeloid leukaemia transformation was 17·8 months (IQR 8·6–36·8; 95% CI 13·6–23·6) in the azacitidine group compared with 11·5 months (4·9–not reached; 8·3–14·5) in the conventional care group (HR 0·50, 95% CI 0·35–0·70; p<0·0001). Results from the investigator preselection subgroup showed a significant difference in time to acute myeloid leukaemia trans-

	BSC only (n=222)				Low-dose cytarabine (n=94)				Intensive chemotherapy (n=42)			
	Azacitidine (n=117)	BSC (n=105)	HR (95%CI)	p value	Azacitidine (n=45)	Low-dose cytarabine (n=49)	HR (95%CI)	p value	Azacitidine (n=17)	Intensive chemotherapy (n=25)	HR (95%CI)	p value
Overall survival (months)	21·1 (10·5–NR)	11·5 (5·7–NR)	0·58 (0·40–0·85)	0·0045	24·5 (8·4–34·7)	15·3 (4·9–25·8)	0·36 (0·20–0·65)	0·0006	25·1 (10·0–NR)	15·7 (8·2–24·1)	0·76 (0·33–1·74)	0·51
Time to transformation to AML (months)	15·0 (8·8–27·6)	10·1 (3·9–19·8)	0·41 (0·27–0·63)	<0·0001	15·0 (7·3–25·5)	14·5 (4·9–19·2)	0·55 (0·28–1·11)	0·097	23·1 (6·4–25·4)	10·7 (4·6–15·4)	0·48 (0·16–1·45)	0·19

Data are median (IQR). Hazard ratios calculated with stratified Cox proportional hazards model adjusted for treatment, subgroup, Eastern Cooperative Oncology Group performance status, lactate dehydrogenase, haemoglobin, number of previous red-blood-cell transfusions, and presence or absence of cytogenetic –7/del(7q) abnormality. No subgroup-by-treatment interactions were significant (p>0·20). BSC=best supportive care. NR=not reached. HR=hazard ratio. AML=acute myeloid leukaemia.

Table 2: **Overall survival and time to progression to acute myeloid leukaemia comparison for groups according to investigator preselection**

	Total ITT (n=358)			BSC only (n=222)			Low-dose cytarabine (n=94)			Intensive chemotherapy (n=42)		
	Azacitidine (n=179)	CCR (n=179)	p value*	Azacitidine (n=117)	BSC (n=105)	p value*	Azacitidine (n=45)	Low-dose cytarabine (n=49)	p value*	Azacitidine (n=17)	Intensive chemotherapy (n=25)	p value*
Haematological response												
Any remission	51 (29%)	21 (12%)	0·0001	32 (27%)	5 (5%)	<0·0001	14 (31%)	6 (12%)	0·042	5 (29%)	10 (40%)	0·53
Complete remission	30 (17%)	14 (8%)	0·015	14 (12%)	1 (1%)	0·0008	11 (24%)	4 (8%)	0·047	5 (29%)	9 (36%)	0·75
Partial remission	21 (12%)	7 (4%)	0·0094	18 (15%)	4 (4%)	0·0058	3 (7%)	2 (4%)	0·67	0	1 (4%)	1·00
Stable disease	75 (42%)	65 (36%)	0·33	52 (44%)	41 (39%)	0·50	15 (33%)	18 (37%)	0·83	8 (47%)	6 (24%)	0·18
Haematological improvement†												
Any improvement	87/177 (49%)	51/178 (29%)	<0·0001	57/115 (50%)	32/105(31%)	0·0058	24/45 (53%)	12/48 (25%)	0·0061	6/17 (35%)	7/25 (28%)	0·74
Major erythroid improvement	62/157 (40%)	17/160 (11%)	<0·0001	39/100(39%)	8/96 (8%)	<0·0001	19/43 (44%)	4/41 (10%)	0·0005	4/14 (29%)	5/23 (22%)	0·70
Major platelet improvement	46/141 (33%)	18/129 (14%)	0·0003	27/89 (30%)	8/78 (10%)	0·0020	14/37 (38%)	6/31 (19%)	0·12	5/15 (33%)	4/20 (20%)	0·45
Major neutrophil improvement	25/131 (19%)	20/111 (18%)	0·87	13/85 (15%)	13/66 (20%)	0·52	9/33 (27%)	3/28 (11%)	0·12	3/13 (23%)	4/17 (24%)	1·00

Data are number (%) or number with improvement/number with data (%). Haematological response and improvement based on International Working Group 2000 criteria for myelodysplastic syndromes.[12] CCR=conventional care regimen. BSC=best supportive care. *p value from Fisher's exact test for comparing patients with response between the azacitidine group and the combined group of CCR, or within investigator preselection, between azacitidine and the individual CCR. †Haemotological improvement can include complete and partial remission.

Table 3: **Haematological response and improvement by treatment group and investigator preselection**

	Total ITT (n=358)		BSC only (N=222)		Low-dose cytarabine (N=94)		Intensive chemotherapy (N=42)	
	Azacitidine (n=179)	Conventional care (n=179)	Azacitidine (n=117)	BSC (n=105)	Azacitidine (n=45)	Low-dose cytarabine (n=49)	Azacitidine (n=17)	Intensive chemotherapy (n=25)
Deaths	82 (46%)	113 (63%)	53 (45%)	66 (63%)	20 (44%)	31 (63%)	9 (53%)	16 (64%)
Deaths during first 3 months* of treatment	20 (11%)	16 (9%)	13 (11%)	9 (9%)	5 (11%)	7 (14%)	2 (12%)	0
Safety population	175	165	114	102	45	44	16	19
Discontinuation before study completion due to haematological adverse events†	8 (5%)	4 (2%)	3 (3%)	2 (2%)	4 (9%)	2 (5%)	1 (6%)	0
Grade 3 or 4 toxicity‡								
Neutropenia	159 (91%)	126 (76%)	104 (91%)	70 (69%)	40 (89%)	39 (89%)	15 (94%)	17 (90%)
Thrombocytopenia	149 (85%)	132 (80%)	93 (82%)	72 (71%)	42 (93%)	42 (96%)	14 (88%)	18 (95%)
Anaemia	100 (57%)	112 (68%)	62 (54%)	67 (66%)	29 (64%)	34 (77%)	9 (56%)	11 (58%)
Baseline grade 0–2 progressed to grade 3 or 4 during treatment‡								
Neutropenia	67/80 (84%)	46/76 (61%)	45/53 (85%)	22/46 (48%)	14/18 (78%)	19/24 (79%)	8/9 (89%)	5/6 (83%)
Thrombocytopenia	72/97 (74%)	68/94 (72%)	49/69 (71%)	29/54 (54%)	17/20 (85%)	29/30 (97%)	6/8 (75%)	10/10 (100%)
Anaemia	84/156 (54%)	83/130 (64%)	52/103 (51%)	48/79 (61%)	25/40 (63%)	28/37 (76%)	7/13 (54%)	7/14 (50%)

Data are number (%) or number/number with data (%). *3 months = 91 days. † Study completion defined as 12 months after the last patient was randomised. ‡National Cancer Institute's Common Toxicity Criteria toxicities based on laboratory data.

Table 4: **Deaths, discontinuations, and grade 3 or 4 haematological toxicity by treatment group and investigator preselection**

formation for azacitidine versus best supportive care. Time to progression to acute myeloid leukaemia did not differ significantly in the comparisons of azacitidine with either low-dose cytarabine or intensive chemotherapy (table 2).

The proportion of patients with complete and partial remission was significantly higher in the azacitidine group than in the conventional care group (table 3). In the investigator preselection analysis, the proportion of patients with complete remission on azacitidine was significantly higher than with either best supportive care or low-dose cytarabine but not higher than with intensive chemotherapy (table 3). The proportion of patients with partial remission with azacitidine was higher than that with best supportive care, but no higher than with the other two treatments. Time to disease progression, relapse after complete or partial remission, and death were significantly longer in the azacitidine group (median 14·1 months, IQR 4·2–27·6) than in the conventional care group (8·8 months, 3·8–not reached; log-rank p=0·047). The proportions of erythroid and platelet improvements were higher in the azacitidine group than in the conventional care group (table 3), but there was no significant difference in the frequency of major neutrophil improvement between the two treatment groups. Duration of haematological response (complete and partial remission and any haematological improvement) was significantly longer in the azacitidine group (median 13·6 months, IQR 5·9–26·4; 95% CI 10·1–16·3) than in the CCR group (5·2 months; 2·9–12·2; 4·1–9·7; log-rank p=0·0002). Median duration of complete plus partial remission in the azacitidine group was 3·2 months (IQR 2·2–4·4; 95% CI 2·4–4·2) versus 3·0 months (2·1–4·0; 2·1–4·0; log-rank p=0·48) in the conventional care group. 50 (45%) of 111 patients (95% CI 35·6–54·8%) who were dependent on red-blood-cell transfusions at baseline in the azacitidine group became transfusion independent compared with 13 (11·4%) of 114 (6·2–18·7) in the conventional care group (p<0·0001).

The rate of infections treated with intravenous antimicrobials per patient year in the azacitidine group was 0·60 (95% CI 0·49–0·73) compared with 0·92 (0·74–1·13) in the conventional care group (relative risk 0·66, 95% CI 0·49–0·87; p=0·0032). There was a significant interaction of treatment by investigator preselection for the rate of infection (p=0·0004). In the investigator preselection analysis, per-patient-year rates were similar when comparing azacitidine (0·66) and best supportive care (0·61; relative risk 1·09, 95% CI 0·74–1·65; p=0·69), but significantly lower with azacitidine (0·44) compared with low-dose cytarabine (1·0; 0·44, 0·25–0·86; p=0·017) and with azacitidine (0·64) versus intensive chemotherapy (2·3; 0·28, 0·13–0·60; p=0·0059).

The most common grade 3–4 events were peripheral blood cytopenias for all treatments (table 4). The most common treatment-related non-haematological adverse events included injection site reactions with azacitidine, and nausea, vomiting, fatigue, and diarrhoea with azacitidine, low-dose cytarabine, and intensive chemotherapy. Treatment discontinuations before study completion in the azacitidine group compared with the conventional care group were mostly related to haematological adverse events (table 4). Table 4 lists the discontinuations because of haematological adverse events by investigator preselection.

During the first 3 months of treatment, deaths occurred in 20 (11%) of 179 patients in the azacitidine group and 16 (9%) of 179 in the conventional care group (table 4). These deaths were primarily attributed to underlying

disease (sepsis or bleeding) although four in the azacitidine group (two from sepsis and two from bleeding), and one in the conventional care group (receiving low-dose cytarabine) from cerebral ischaemia were probably related to treatment. Table 4 shows deaths in the first 3 months of treatment by investigator preselection.

Discussion

Treatment with azacitidine prolongs overall survival and lowers the risk of progression to acute myeloid leukaemia in patients with higher-risk myelodysplastic syndrome compared with treatment with conventional care regimens.

The previous CALGB trial[16] included a heterogeneous population of patients, best-supportive care as the only comparator, and a crossover design, and 53% of patients who received best-supportive care subsequently received azacitidine. Our study aimed to include only patients with higher-risk disease (87% were intermediate-2 or high on the international prognosis scoring system), and did not allow crossover. Furthermore, in the absence of a standard of care for the control regimen in higher-risk myelodysplastic syndromes, and with differing national, regional, institutional, or consensus guidelines,[24,25] this trial compared azacitidine treatment with a control arm including the three most common treatments for higher-risk myelodysplastic syndromes over the past two decades (best supportive care, low-dose cytarabine, and intensive chemotherapy).[6–15,17] Randomisation allowed valid comparisons within investigator preselected subgroups. Patients enrolled in the study were representative of those with higher-risk myelodysplastic syndrome in demographic characteristics, presenting signs and symptoms, and subtypes on the FAB classification. The proportions of patients selected to the best supportive care, low-dose cytarabine, and intensive chemotherapy groups were consistent with treatment practices (Germing U, Heinrich-Heine University, Dusseldorf, Germany, personal communication).[26]

Median overall survival in the azacitidine group exceeded that in the conventional care group by 9·4 months with a 2-year survival rate that was nearly doubled. The survival benefit with azacitidine was seen across all prognostic subgroups analysed, including those patients with poor, intermediate, and good cytogenetics according to the international prognosis scoring system. Patients with numerical or structural abnormalities of chromosome 7, who have a particularly poor outcome with traditional management strategies,[17,27–29] had overall survival improvement with azacitidine.

The survival advantage in the azacitidine group was observed early in the treatment course compared with the conventional care group, with separation of the Kaplan-Meier survival curves occurring after about 3 months of treatment, corresponding to completion of about three cycles of azacitidine treatment by most patients. The median number of azacitidine treatment cycles was nine, suggesting that long-term treatment might give the best survival benefit.

Comparisons with the supportive investigator preselection analysis showed that treatment with azacitidine was associated with a significant improvement in overall survival compared with low-dose cytarabine or best supportive therapy. Previous studies of low-dose cytarabine in high-risk myelodysplastic syndrome or acute myeloid leukaemia have reported low proportions of patients with response and poor survival,[30] particularly in patients with unfavourable cytogenetics.[17,31,32] A previous trial that compared treatment with one cycle of low-dose cytarabine with best supportive care showed no survival differences.[33] Although our trial was designed to maximise the potential of each treatment strategy by continuing treatment until evidence for disease progression, the median number of cycles with low-dose cytarabine was four and a half because of a combination of poor response, disease progression, and unacceptable toxicity.

The difference in median overall survival between the azacitidine and intensive chemotherapy groups was not statistically significant, possibly because of the small number of patients in this analysis. The proportion of patients with complete remission with intensive chemotherapy (40%) was in the range of published reports of myelodysplastic syndrome[9–15] and higher than that observed with azacitidine in this and the CALGB studies.[16] In patients who are candidates for allogeneic stem-cell transplantation and have a clear excess of marrow blasts (especially those with refractory anaemia with excess blasts in transformation, now classified as acute myeloid leukaemia in the WHO classification), intensive chemotherapy might be preferred to azacitidine before transplantation to provide better and more rapid reduction in marrow blast percentage. However, the value of reducing the blast percentage before transplantation in myelodysplastic syndromes is still disputed.[12] Furthermore, intensive chemotherapy is associated with high proportions of complete remission in myelodysplastic syndrome only in the absence of an unfavourable karyotype,[13,14] and patients transplanted after failure of intensive chemotherapy have a very poor outcome after transplantation.[12] On the basis of results achieved with azacitidine in patients with unfavourable karyotype in the present study, this drug is being investigated before transplantation in patients with myelodysplastic syndrome with an excess of marrow blasts and unfavourable karyotype.[34]

In the investigator preselection analysis, grade 3 and 4 neutropenia was more common in patients receiving azacitidine, low-dose cytarabine, and intensive chemotherapy than in those receiving best supportive care. Thrombocytopenia was also more common with azacitidine than with best supportive care, but less common than with low-dose cytarabine and intensive chemotherapy. Despite the higher frequency of

thrombocytopenia and neutropenia observed with azacitidine compared with best supportive care, the frequency of haemorrhagic complications and infection was similar for both treatments. Risk of infection requiring intravenous antimicrobials was a third lower in the azacitidine group than in the conventional care group.

Finally, mechanisms of the activity of azacitidine in myelodysplastic syndromes are not fully known. Aberrant DNA hypermethylation has been implicated in the progression of myelodysplastic syndromes, and DNA-methyltransferase inhibitors, such as azacitidine, undo hypermethylation and restore normal transcription of tumour suppressor genes.[35,36] Additional mechanisms of action of azacitidine in myelodysplastic syndromes are, however, probable, including a certain degree of tumour-cell apoptosis. Research to identify these mechanisms is underway.[35,37,38]

This trial was international and multicentre in design with 79 investigative sites in 15 countries. In the comparison of azacitidine with the three most common treatments in higher-risk myelodysplastic syndromes, including two active treatments, treatment decisions were made in light of different treatment practices influenced by regional, national, and local guidelines and consensus criteria. For these reasons, the results are applicable to the improvement of the treatment of myelodysplastic syndromes internationally. Ultimately, intensive chemotherapy might remain the appropriate treatment in some situations in higher-risk myelodysplastic syndromes, especially before allogeneic stem-cell transplantation in candidates for this procedure who have an excess of marrow blasts without an unfavourable karyotype.

Increased survival time is the primary goal of treatment for patients with higher-risk myelodysplastic syndromes. However, with the exception of allogeneic haemopoietic stem-cell transplantation, which is suitable for only a few patients with myelodysplastic syndrome,[27] no previous treatment strategies have shown a significant overall survival benefit. The results of this study indicate that azacitidine significantly lengthens overall survival and changes the natural history of myelodysplastic syndrome in patients with higher-risk disease.

Contributors

PF, GJM, EH-L, AL, SDG, JFS, JMB, JBy, JBa, LZ, DM, CLB, LRS designed the trial. PF, GJM, EH-L, VS, CF AG, RS, NG, GS, and JFS did the research. DM of Celgene analysed the data. PF wrote the paper. All authors provided review and editing of the manuscript.

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Conflicts of interest

PF has participated in advisory board meetings for Celgene, Roche, Amgen, GlaxoSmithKlein, Merck, Novartis, Johnson and Johnson, and Cephalon. GJM has served as an advisory board member and consultant for Celgene, Amgen, and Genzyme, and as an advisory board member for Pharmion (now Celgene) and Johnson and Johnson. EH-L has participated in advisory board meetings for Celgene and Amgen and has given paid testimony for Celgene. VS has received honoraria from Celgene, Novartis, and Johnson and Johnson for lecturing. CF has no conflicts of interest. AG is a consultant to Celgene and participates on their speakers' bureau. RS has nothing to disclose. NG has received honoraria for lecturing for Novartis, Roche, Celgene, and Janssen-Cilag and research support from Novartis, and Celgene. GS is a consultant for Celgene. AL has received honoraria from Celgene. SDG is a consultant for and owns stock in Celgene. JFS has participated on an advisory board for and has received honoraria from Celgene; he was a member of an advisory board and speaker's bureau for Pharmion (now Celgene) and he received honoraria from them. JMB is a consultant for Celgene, Novartis, and Johnson and Johnson and is a member of a speakers' bureau for Celgene. JBy is a consultant for Celgene. JBa, LZ, DM, and CLB are employees of Celgene and own stock in the company. LRS has received research funding and honoraria from Celgene.

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Demethylating agents in myeloid malignancies

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Purpose of review

Two demethylating agents are approved in myelodysplastic syndromes (MDS): 5-azacitidine and 5-aza-2′-deoxycitidine (decitabine). These drugs are structurally related and induce DNA hypomethylation. Aberrant DNA methylation is associated with gene silencing. It is proposed that hypomethylating agents work by inducing reexpression of epigenetically silenced genes. Here, we provide an up-to-date summary of the clinical experience with these drugs.

Recent findings

5-Azacitidine and decitabine were approved in the United States based on clinical responses, but no effect on survival was documented. Recent results from a phase III study have indicated that treatment of patients with higher risk MDS with 5-azacitidine results in significant improvement in overall survival. Results of a randomized survival study of decitabine should be available in 2008. Reports of combination epigenetic therapies (a hypomethylating agent with a histone deacetylase inhibitor) indicate that these have significant activity in patients with MDS/acute myelogenous leukemia. Randomized studies are testing the concept that the combinations are superior to single-agent therapy.

Summary

Demethylating agents are the standard of care for patients with higher risk MDS and the only agent known to improve the natural history of MDS. Further work in new combination therapies may result in further advances in the care of patients with MDS.

Keywords

DNA methylation, epigenetics, leukemia

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Introduction

The term demethylating agents refers to a group of chemotherapeutic agents with the capacity, both *in vitro* and *in vivo*, to induce transient DNA hypomethylation. DNA methylation refers to the addition of a methyl group to a CpG site [1]. These sites cluster together in areas known as CpG islands and are frequently localized in the proximity of key gene regulatory regions such as gene promoters. DNA methylation, both aberrant and physiologic, of these areas can result in gene silencing and in the equivalent of the physical inactivation, due to either mutations or deletions, of tumor suppressor genes [2•]. At the present time, two hypomethylating agents are approved in the United States and are widely used in Europe and the rest of the world: 5-azacitidine [3] and 5-aza-2′-deoxycitidine [4] (decitabine). These two agents have significant activity in patients with higher risk myelodysplastic syndromes (MDS). Recently, 5-azacitidine has been reported to improve survival in patients with higher risk MDS in a randomized phase III study [5••]. In this manuscript, we will provide an up-to-date review of the results with these two agents in MDS and other diseases.

Single-agent decitabine

Decitabine is a nucleoside analogue that inhibits DNA methyltransferase (DNMT) activity resulting in global and gene-specific DNA hypomethylation [6]. The clinical development of this agent goes back several decades [7]. Initially, decitabine was used in several small clinical trials in acute myelogenous leukemia (AML) and higher risk MDS and eventually chronic myelogenous leukemia (CML). At that time, the development of decitabine focused on its use at the highest possible dose, in a way similar to the use of cytarabine (ara-C), a drug to which it is structurally related. It should be noted that ara-C has no hypomethylating activity. Initial clinical trials with decitabine did not show any significant superiority compared with ara-C and the development of decitabine was halted until several groups reported the importance of aberrant DNA methylation as a critical event in cancer, and that this process could be reversed by

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Table 1 Results with single-agent 5-aza-2′-deoxycitidine

Study	Initial 3-day schedule [9]	Randomized 3-day [10]	5 day schedule [11••]
Phase	II	III	II
N	66	89	95
CR	13 (20)	8 (9)	32 (34)
PR	3 (4)	7 (8)	1 (1)
HI	16 (24)	12 (13)	13 (13)
OR	32 (48)	27 (30)	46 (48)

hypomethylating agents, particularly when using these drugs at lower concentrations [8]. This led to the re-emergence of an interest in performing clinical trials with these compounds in MDS and AML [9]. The initial studies with decitabine were conducted by the European Organization for Research and Treatment of Cancer (EORTC) in Europe. The schedule studied in those trials was a dose of decitabine of 15 mg/m^2 intravenously (i.v.) infused over 4 h three times a day daily for 3 days with courses repeated every 6 weeks. The three times a day schedule was an effort to provide the closest to a continuous infusion. Continuous infusions with decitabine and 5-azacitidine are impractical due to the fact that the drugs decay in solution. The 6 weeks schedule was implemented due to a concern of potential prolonged myelosuppresion with more frequent administration rates. Results of initial trials with this schedule of decitabine included an overall response rate (ORR) of 48% (Table 1). On the basis of this data, this schedule (that we are going to refer from here on as the '3-day schedule') was studied in the United States in a randomized phase III trial that led to the approval of decitabine [10]. Results are also summarized in Table 1. Decitabine administration resulted in more clinical responses compared with supportive care (30 versus 7%). Patients treated with decitabine had a trend toward a longer median time to AML or death compared with patients treated with supportive care alone (12.1 months versus 7.8 months; $P=0.16$). Patients who were treatment naive (12.3 months vs. 7.3 months; $P=0.08$), or had an International Prognostic Scoring System (IPSS) score of intermediate-2/high risk (12.0 months vs. 6.8 months; $P=0.03$), or had de-novo MDS (12.6 months vs. 9.4 months; $P=0.04$) had a trend toward improved survival. No differences in toxicity could be observed between the decitabine arm and the control group.

Although the results of these initial studies are of great relevance, it was clear that alternative schedules that did not require hospital admission with more activity were needed. Because lower concentrations of decitabine are more efficient as hypomethylating agents (J.P. Issa, personal communication), a phase I study of decitabine was conducted studying a daily i.v. schedule [12]. Dose escalation was performed not only on the daily dose but also on the number of days of drug administration. This study was open to both patients with acute and chronic leukemias. Doses of decitabine ranged from 5 mg/m^2 daily for 10 days to 15 mg/m^2 daily for 20 days. In this study, decitabine was infused i.v. over 1 h. The ORR was 32% and the maximal tolerated dose was not defined. Of importance, an intermediate dose schedule (15 mg/m^2 daily × 10 dose/schedule) was associated with the highest response rate (five out of six patients treated at that dose, 83%). Indeed, clinical activity decreased at doses above and below this dose level. Because of these results, a second cohort of 11 patients was treated at the 15 mg/m^2 daily × 10 dose/schedule with an ORR of 55%. This confirmed the activity of this intermediate dose level of decitabine. Because of the clinical activity of this daily schedule of decitabine, a randomized phase II study of three different schedules of daily decitabine was conducted [11••]. The total dose of decitabine in each arm was 100 mg/m^2. The randomization favored the 20 mg/m^2 daily × 5 dose/schedule as the most active. With this schedule of decitabine drug-related mortality was approximately 5%. Main toxicities were myelosuppresion and its associated complications that resulted in admission to the hospital in 66% of patients. The median overall survival (OS) was 19 months. Median number of courses administered was 6+ (1–18+). To assess the effect of these newer schedule of decitabine (5-day schedule herein), an analysis was performed comparing the survival of patients with MDS treated with decitabine in this study with that of a contemporary cohort of patients treated with an ara-C containing regimen at MD Anderson Cancer Center (MDACC) [13]. This study indicated that treatment with decitabine was associated with improved survival compared with prior experience with ara-C-based programs. This was despite the fact the complete remission rates were lower with decitabine as compared with intensive chemotherapy programs. This is probably due, in part, to the very low induction mortality rate observed with decitabine [13]. This data represented a change in practice, as we may accept a lower response rate if induction mortality is also significantly lower, and translates into better survival.

The results of this trial confirmed the activity of decitabine in MDS and indicated that the 5-day schedule is currently the optimal way to administer this agent. An update of the 5-day schedule from MDACC was presented at ASH 2007 [14]. Overall 36 patients (39%) achieved a complete remission, and 75 (81%) had a response by International Working Group (IWG) criteria. The median number of cycles administered was 8+. The median time to response was 2.3 months, and the median duration of complete remission was 14 months (range 3 to 16+ months). To confirm these results, a multicenter single arm phase II study was conducted in North America using the 5-day schedule. The ORR reported was 43% with a median of five courses of therapy administered [15]. Final results of this study are

to be published but the data presented demonstrated the activity and safety of the 5-day schedule. Other important data not currently available are the results of a 'survival study' conducted in Europe comparing decitabine versus best supportive care. The results of this study are expected during 2008.

Single-agent 5-azacitidine

5-Azacitidine is another nucleoside analogue structurally related to decitabine [16]. In contrast to decitabine, 5-azacitidine is a ribose structure that is incorporated into RNA and requires the activity of ribonucleotide reductase (RNR) to be incorporated into DNA and to exert its hypomethylating effect. It should be noted that RNR is the target of hydroxyurea and, therefore, the concomitant use of hydroxyurea and 5-azacitidine should be avoided [17]. Initial studies with 5-azacitidine were performed by the Cancer and Leukemia Group B (CALGB) and demonstrated that with either an i.v. or subcutaneous route of administration for 7 days, 5-azacitidine had activity in MDS and AML [18]. On the basis of the data from a subcutaneous study, a randomized phase III cross-over study was conducted by the CALGB comparing 5-azacitidine (75 mg/m^2 subcutaneous daily $\times$ 7) versus supportive care [3] in patients with MDS. Results are shown in Table 2. In summary, the study demonstrated an ORR of 60% with 5-azacitidine compared with 5% with supportive care. Patients in the cross-over arm (those on the supportive care arm that then went to receive 5-azacitidine) had an ORR of 47%, thus confirming the results with 5-azacitidine. Median OS on the 5-azacitidine was 20 months compared with 14 for the supportive care arm. This was not significant due to the cross-over design of the study. This study (CALGB 9221) led to the approval of 5-azacitidine in the United States for patients with MDS [3]. Because this study failed to demonstrate a survival effect [5••], a second randomized study was performed to study the effect of 5-azacitidine on survival. The results of this study were reported at the ASH 2007 meeting [5••]. In that study, 358 patients with higher risk MDS (int-2 and high risk by IPSS) received either 5-azacitidine or 'conventional care'. Conventional care could include supportive care, low-dose ara-C or a traditional '7 + 3' induction programme. Response rates with 5-azacitidine are shown in Table 2.

Table 2 Results with single-agent 5-azacitidine

Study	CALGB 9221 [3]	Updated CALGB 9221 [18]	Survival study [5••]
Phase	III	III	III
N	99	99	179
CR	7 (7)	10 (10)	30 (17)
PR	16 (16)	1 (1)	21 (12)
HI	37 (37)	36 (36)	87 (49)
OR	60 (60)	47 (47)	138 (78)

CALGB, Cancer and Leukemia Group B.

Importantly, a significant effect was observed on survival in patients that received 5-azacitidine versus the other interventions (hazard ratio, 0.58; 95% confidence interval 0.43, 0.77). This study still needs to be presented in article form, but one of the key issues was the fact that patients with alterations of chromosome 7 were one of the groups that derived the most benefit from therapy. This data demonstrates the capacity of 5-azacitidine to alter the natural history of patients with MDS. This is demonstrated by the capacity of 5-azacitidine to potentially double the survival at 2 years in patients with higher risk disease (24.4 months on 5-azacitidine versus 15 months on conventional care) and establishes the role of 5-azacitidine as standard of care in this patient population.

Combination strategies

Traditionally, achievement of complete remission has been a prerequisite for improved survival. From that perspective, the use of hypomethylating agents has been associated with a relative low rate of complete remission rates (Tables 1 and 2). Several groups have attempted to increase the response rate with hypomethylating-based therapy by developing combinations. Because epigenetic manipulation with hypomethylating agents can lead to reactivation of aberrantly silenced genes and, therefore, targets, multiple rational sequential combinations are possible. From a mechanistic perspective, the most widely studied combination is that of a hypomethylating agent and a histone deacetylase (HDAC) inhibitor [19,20]. Initial studies with trichostatin, and phenylbutyrate led to combinations with valproic acid, once this drug was discovered to have HDAC inhibitory activity [21,22]. Valproic acid is a fatty acid chain derivative traditionally used to treat epilepsy and other neurological disorders [23]. Studies performed in Germany indicated that single-agent valproic acid has activity in lower risk MDS [24]. On the basis of these at least three studies have been reported with the combination of 5-azacitidine or decitabine with valproic acid [25,26•,27] (Table 3). These studies with valproic acid have been mainly phase I or early phase II trials in patients with AML and higher risk MDS. Reported ORR (CR/CRps) ranged from 20 to 50%. In patients with previously untreated disease, the response rate was approximately 50%. Of importance, time to response has been consistently one course (ranging from one to three courses of therapy) and appears to be faster than the four to six courses required with single-agent 5-azacitidine for initial response (add two more courses for best response), at least with 5-azacitidine [28]. Induction mortality has also been very low, generally less than 5%. Main toxicity has been neurological related to valproic acid use. An important issue has been the optimal dose/schedule of valproic acid. In-vitro studies had indicated that doses of at least 1 mmol/l of valproic acid are required for induction of

Table 3 Results of combination studies with valproic acid

Study	Decitabine and valproic acid [25]	Decitabine and valproic acid in untreated patients [25]	5-azacitidine and valproic acid [26•]	5-azacitidine and valproic acid in untreated patients [26•]
Phase	I/II	III	I/II	I/II
N	53	99	53	33
CR	10 (19)	10 (10)	12 (22)	11 (33)
PR	2 (4)	1 (1)	3 (6)	3 (9)
HI	NA	36 (36)	7 (13)	3 (9)
OR	12 (23)	47 (47)	22 (41)	17 (51)

histone acetylation and that induction of cell death is proportional to the concentration of the drug used [29]. Indeed, in two studies of valproic acid at MDACC [25,26•], patients that responded to the combination of valproic acid with either decitabine or 5-azacitidine tended to have the higher valproic acid levels in blood, recapitulating the in-vitro data and arguing for the use of short high intensity courses of valproic acid, instead of prolonged lower dose schedule proposed by other investigators [24]. This is also supported by the fact that toxicity with valproic acid is transient and that there is no good correlation between dose and levels, and blood levels and toxicity [26•].

The present phenomenon of dose response with valproic acid observed in the studies described above supports the notion that replacing valproic acid by more potent HDAC inhibitors [20] may result in very active clinical combinations. Such studies are ongoing with both 5-azacitidine and decitabine with several HDAC inhibitors. One such study is the combination of 5-azacitidine and MGCD0103 [30]. MGCD0103 is a class 1 specific HDAC inhibitor with activity in AML and potentially MDS [31]. For the combination study, patients with relapse/refractory or de-novo MDS/AML were eligible. A dose of MGCD0103 of 90 mg oral three times a week was found to be well tolerated in combination with 5-azacitidine. Response rates were approximately 50% in the group of patients that had not received prior therapy. Median time to response was also one cycle (range one to three). Importantly, median OS has not been reached in patients that responded to therapy. This study serves as the basis for an ongoing randomized study comparing 5-azacitidine with or without MGCD0103. This type of study is crucial to demonstrate the concept that the combination is better than single-agent. Several other studies (decitabine ±valproic acid and 5-azacitidine ±MS-275) are ongoing.

Other combinations include 5-azacitidine with lenalidomide [32] or TNF-α modulation [33] and the combination of decitabine or 5-azacitidine with mylotarg [34]. Although these are small early pilot studies and the data limited, these experiences are of significant interest. In particular, the combination with mylotarg is important in view of the early results with this combination (a complete remission rate of 75% in 13 patients with elderly AML) as well as the fact that mylotarg has activity as a single-agent in AML. Sequence seems to be important for the mylotarg and it should be administered at the end of the administration of the hypomethylating agent. Other combinations include the use of priming with growth factor support and with the use of thrombomimetic agents.

Other indications

Hypomethylating agents may have activity in solid tumors malignancies, lymphomas and other leukemias. For instance, decitabine has activity in CML in all phases of the disease, either alone or in combination with imatinib [35]. These studies were prior to the development of second-generation tyrosine kinase inhibitors, such as nilotinib or dasatinib, but demonstrated that this type of epigenetic manipulation may have a role in patients with advanced phase CML. Hypomethylating agents are also being tested in chronic lymphocytic leukemia and acute lymphocytic leukemia. It is too early to say whether this will have activity in this setting. In view of the activity of hypomethylating agents in MDS, it will be expected that they will also be active in AML, especially in older patients with poor risk characteristics, such as poor cytogenetics, who are not usually candidates for intensive chemotherapy. Although there is data in this setting with both 5-azacitidine and decitabine, two studies with decitabine (one by the EORTC using the 3-day schedule) and the other randomizing against low-dose ara-C are ongoing and could result in a new indication for decitabine. Another approach is the use of hypomethylating agents as maintenance type of strategy in patients in remission after more intense type of therapy. Data from the Nordic group was presented at ASH 2007. In a group of patients with higher risk MDS or AML that received '7 + 3' type induction therapy followed by maintenance with 5-azacitidine the OS was 17 months in patients that received 5-azacitidine maintenance therapy [36]. Other studies with both decitabine and 5-azacitidine are exploring this issue that may have significant implications for patients with AML.

Conclusion

The development of the hypomethylating agents has resulted in the first group of therapies to provide a clear and consistent benefit to patients with MDS. It is

possible that this could also translate into a benefit in AML, other leukemias and potentially solid tumors and lymphomas. Although the data with 5-azacitidine on survival [5••] is probably one of the most important in MDS, it is obvious that we are far from the results obtained with the tyrosine kinase inhibitors in CML. It is expected that more active compounds with more clinical activity will translate into greater survival benefits. Therefore, the development of new more potent hypomethylating agents is of importance. Oral formulations [37] are under development but their pharmacokinetic characteristics need to be fully understood. Perhaps the development of new more potent HDAC inhibitors and their combinations could improve on the therapeutic activity of these forms of epigenetic therapy.

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

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Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 734).

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The Acquisition of *hMLH1* Methylation in Plasma DNA after Chemotherapy Predicts Poor Survival for Ovarian Cancer Patients

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ABSTRACT

Aberrant epigenetic regulation, such as CpG island methylation and associated transcriptional silencing of genes, has been implicated in a variety of human diseases, including cancer. Methylation of genes involved in apoptosis, including the DNA mismatch repair (MMR) gene *hMLH1*, can occur in tumor models of resistance to chemotherapeutic drugs. However, the relevance for acquired resistance to chemotherapy of patients' tumors remains unsubstantiated. Plasma DNA from cancer patients, including those with ovarian cancer, often contains identical DNA changes as the tumor and provides a means to monitor CpG island methylation changes. We have examined plasma DNA of patients with epithelial ovarian cancer enrolled in the SCOTROC1 Phase III clinical trial for methylation of the *hMLH1* CpG island before carboplatin/taxoid chemotherapy and at relapse. Methylation of *hMLH1* is increased at relapse, and 25% (34 of 138) of relapse samples have *hMLH1* methylation that is not detected in matched prechemotherapy plasma samples. Furthermore, *hMLH1* methylation is significantly associated with increased microsatellite instability in plasma DNA at relapse, providing an independent measure of function of the MMR pathway. Acquisition of *hMLH1* methylation in plasma DNA at relapse predicts poor overall survival of patients, independent from time to progression and age (hazard ratio, 1.99; 95% confidence interval, 1.20–3.30; $P = 0.007$). These data support the clinical relevance of acquired *hMLH1* methylation and concomitant loss of DNA MMR after chemotherapy of ovarian cancer patients. DNA methylation changes in plasma provide the potential to define patterns of methylation during therapy and identify those patient populations who would be suitable for novel epigenetic therapies.

INTRODUCTION

Transcriptional silencing of proapoptotic genes, including the DNA mismatch repair (MMR) gene *hMLH1*, due to aberrant CpG island methylation has been implicated in acquired resistance to chemotherapeutic drugs *in vitro* (1, 2). A major limitation in confirming the clinical relevance of drug resistance mechanisms is the difficulty in obtaining tumor biopsies after initial treatment, at a time when resistant subpopulations may be more apparent. However, plasma DNA from cancer patients, including those with ovarian cancer, often contains the same genetic changes as the tumor (3, 4), raising the possibility of using plasma DNA to monitor genetic and epigenetic changes after treatment.

Although relatively chemosensitive, with response rates to primary chemotherapy of 60–80%, the majority of ovarian tumors will recur, leading to failure of treatment using conventional cytotoxic drugs and resulting in an overall 5-year survival for patients with advanced disease of <30% (5). Many reports have been published on potential drug resistance markers in ovarian cancer, derived mainly from the study of acquired resistance in experimental models (6). However, most clinical studies of drug resistance have focused on tumor characteristics at presentation, rather than at relapse. Whereas studies of tumors before chemotherapy are important for identifying prognostic markers and possible mechanisms of intrinsic resistance, they will provide limited information on mechanisms of acquired resistance. Thus, tumors at presentation will be heterogeneous, consisting of chemosensitive and chemoresistant subpopulations, making it difficult to identify the subpopulations that lead to treatment failure of an initially responsive tumor. Because chemotherapy positively selects for resistant subpopulations, analysis of tumors at relapse may allow these subpopulations of cells to become more apparent and will allow mechanisms of acquired rather than intrinsic drug resistance to be identified and analyzed for associations with patient survival.

Due to the difficulties in obtaining tumor samples routinely, especially from patients at relapse, and for ease of sample collection in the context of large, multicenter clinical trials, there has been increasing interest in the use of markers in plasma and serum for the prognostication and monitoring of cancer (3). DNA can be detected in plasma from cancer patients with the same characteristic changes, including CpG island methylation, found in the corresponding tumor (7). In the case of ovarian cancer, such changes have been detected with high specificity and have been suggested as a diagnostic tool (4, 8). DNA methylation is particularly suited for such analysis of

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plasma DNA because sensitive methylation-specific PCR (MSP)-based assays require only small amounts of DNA, and methylation of genes frequently aberrantly methylated in tumors is rarely observed in normal tissue, including peripheral blood mononuclear cell (PBMC) DNA that may be present with tumor DNA in plasma (9).

In vitro studies of ovarian cancer cell lines have implicated loss of DNA damage-dependent apoptotic pathway in acquired resistance to clinically important cytotoxic drugs (10). Methylation of *hMLH1* and loss of a MMR-dependent apoptotic response lead to increased resistance to cisplatin and carboplatin, drugs that are the cornerstone of treatment of ovarian cancer and a wide variety of tumors (2, 11). Restoration of *hMLH1* expression, either by gene transfer or by reversal of epigenetic silencing, leads to increased sensitivity to carboplatin and other cytotoxic agents (12, 13). However, the clinical relevance for acquired resistance to chemotherapy of epigenetic inactivation of proapoptotic genes such as *hMLH1* by DNA methylation remains uncertain. Indeed, methylation of antiapoptotic genes or genes involved in protecting cells from DNA damage can also occur in cancer and could lead to increased drug sensitivity (14). Therefore, identification of epigenetic changes that influence clinical outcome after chemotherapy, as well as allow target validation, will have potential for disease stratification and treatment individualization.

MATERIALS AND METHODS

Patients and Collection of Plasma. In the SCOTROC1 randomized trial, all patients had histologically confirmed epithelial ovarian carcinoma and FIGO (International Federation of Gynecologists and Obstetricians) stage Ic–IV disease with or without cytoreductive surgery at staging laprotomy. All patients gave written informed consent, and appropriate ethical review boards approved the study. Patients were randomized to receive six cycles of either paclitaxel (175 mg/m^2) as a 3-h infusion or docetaxel (75 mg/m^2) as a 1-h infusion in combination with carboplatin AUC5 as 1-h infusion. Ten ml of blood were collected before chemotherapy or at the time of disease progression (relapse) after primary chemotherapy into EDTA tubes, and the plasma was separated from PBMCs before being sent to the Cancer Research UK Beatson Laboratories (Glasgow, United Kingdom). All laboratory analyses were conducted blinded to clinical outcome. Progressive disease was defined as either clinical evidence of progressive disease based on the South Western Oncology Group Solid Tumor Response Criteria or elevated CA125 levels as defined previously (15) and clinical or radiographic findings indicative of progression. Five relapse blood samples were collected at the time of clinically suspected progression, but before progression was verified. More than 90% of the relapse samples were taken <4 weeks after the date of confirmed progression.

DNA Isolation. DNA was extracted from 200 μl of blood and 1 ml of plasma using the QIAmp DNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions, with plasma elute passed through the column an additional four times to increase yield and eluting in a final volume of 200 μl. The mean yield of DNA at presentation was 2.8 μg/ml plasma (SD, 1.7 μg/ml plasma), and at relapse, it was 3.6 μg/ml plasma (SD, 1.8 μg/ml plasma). However, it should be noted that this will represent DNA from lysed normal blood cells as well as tumor DNA.

MSP. MSP was done essentially as described previously (16). Fifty μl of DNA from each serum sample were bisulphite modified using the CpGenome DNA Modification Kit (Serologicals Corp.) according to the manufacturer's instructions. For each modification, a negative control of 100 and 500 ng of human Genomic DNA (Promega) was modified, and positive controls of serial dilutions (1:5, 1:10, 1:20, 1:50, 1:100, 1:200, and 1:500) of CpGenome Universally Methylated human DNA were diluted into human genomic DNA before modification. PCR for hMLH1 CpG island was performed essentially as described previously but with the primer sequences 5′-GGGTTAACGTTAGAAAGGTCG and 5′-CGCTTACGCGTTAAAAATCGC (17). The forward primer was fluorescently labeled with 5′-FAM dye (Applied Biosystems). PCR products were denatured at 95°C for 5 min and electrophoresed for 12 h at 2500 V on a 6% denaturing polyacrylamide gel (Flowgen) using a 373XL Stretch DNA Sequencer (Applied Biosystems). Samples were run with the internal lane size standard GS500XL ROX and analyzed using Genescan 3.1 Analysis Software (Applied Biosystems) to determine the size of the PCR products and the amount of fluorescent signal.

Microsatellite PCR. The choice of microsatellite loci used reflects those used in previous colorectal cancer and ovarian analysis (8, 18). PCR was carried out using primers (synthesized by Oswel DNA Service, Southampton, United Kingdom) specific for six polymorphic DNA microsatellite loci (*Mfd15CA, D2S123, P53, D5S346, D18S69*, and *D18S58*). The forward primer in each case was fluorescently labeled with 5′-FAM or HEX (Applied Biosystems). PCR products were denatured at 95°C for 5 min and electrophoresed for 12 h at 2500 V on a 6% denaturing polyacrylamide gel (Flowgen) using a 373XL Stretch DNA Sequencer (Applied Biosystems). Samples were run with the internal lane size standard GS500XL ROX and analyzed using Genescan 3.1 Analysis Software (Applied Biosystems) to determine the size of the PCR products and the amount of fluorescent signal. Allelic shifts were defined as a shift in the major PCR product(s) by >2 bases of plasma DNA compared with PBMC DNA. Samples with a major peak of PCR products of <30 fluorescence units were not included in the analysis.

Statistics. The change in methylation and microsatellite instability (MSI) status from prechemotherapy to progression was compared in the paired samples using the Wilcoxon signed rank-sum test. The examination of the association between MSI status and methylation status was conducted using Fisher's exact test. Kaplan-Meier methods were used to produce the survival curves in Fig. 4. All causes of death were considered as events. Cox regression techniques were used to analyze associations with survival; in the adjusted analysis, time to progression was included as an unordered categorical variable (<6 months, 6–12 months, >12 months), and age was included as a continuous variable.

RESULTS

***hMLH1* Methylation in Plasma DNA.** We have examined CpG island methylation changes of the *hMLH1* gene in

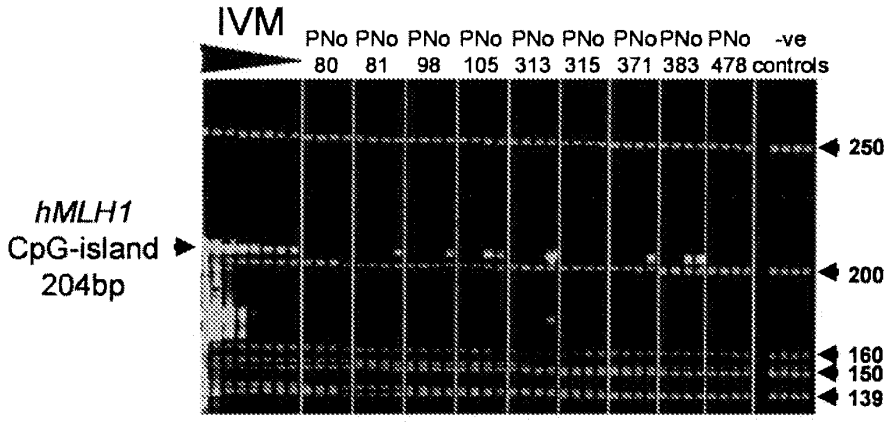

Fig. 1 Fluorescent methylation-specific PCR (MSP) of *hMLH1* CpG island. The *hMLH1* CpG island MSP product (204 bp) is shown in *blue*. The *first eight lanes* show dilutions of *in vitro* methylated (*IVM*) human DNA into peripheral blood mononuclear cell (PBMC) DNA (dilutions of 1:1, 1:5, 1:10, 1:20, 1:50, 1:100, 1:200, and 1:500). The last four lanes show negative controls of 100 and 500 ng of PBMC DNA and two water controls. The MSP products of SCOTROC1 patient number (*PNo*) samples are shown in groups of four and are, in order, DNA from PBMCs prechemotherapy, PBMCs at relapse, plasma prechemotherapy, and plasma at relapse. The sizes (in bp) of the markers (*red*) are shown.

plasma DNA from patients at presentation and at relapse from the SCOTROC1 Phase III clinical trial, a randomized, prospective comparison of paclitaxel-carboplatin *versus* docetaxel-carboplatin as first-line chemotherapy in stage Ic–IV epithelial ovarian cancer. The results of this clinical study have not yet been published, but preliminary analysis has been presented, and at that time, similar response rates, progression-free survival, and overall survival rates were observed for both treatment arms (19). Examining *hMLH1* methylation and MSI in plasma DNA were additional objectives of the trial. Of the first 480 patients enrolled on the SCOTROC1 trial, 351 had relapsed at the time of analysis, and matched lymphocyte and plasma samples before chemotherapy and at relapse, with informed patient consent, were received from 149 of these patients. Samples were removed from analysis if it became clear that the prechemotherapy and relapse samples did not match (*e.g.*, showed divergent microsatellite alleles in PBMC DNA) or that insufficient DNA was recovered; reliable results were available for 138 samples prechemotherapy and 144 samples at relapse (138 paired samples).

We have used MSP with fluorescently labeled primers to detect methylation of the *hMLH1* CpG island (Fig. 1). We have shown previously in ovarian tumor samples that methylation of this CpG island is associated with reduced expression of MLH1 (17) and that reversal of methylation of the CpG island in ovarian cell lines by DNA methyltransferase inhibitors leads to MLH1 reexpression (2). The fluorescent MSP assay is very sensitive, detecting down to a 1:500 dilution of *in vitro* methylated human DNA diluted into human lymphocyte DNA. As shown in Fig. 1, methylation of *hMLH1* is generally not detected in DNA from PBMCs from matched patients before chemotherapy or at relapse using the same DNA concentrations and PCR conditions as used for the plasma DNA. Examples of plasma DNAs positive for *hMLH1* CpG island methylation at prechemotherapy and at relapse are shown for patients 105 and 383, whereas patients 81, 98, 313, and 371 show evidence of *hMLH1* methylation only in the relapse plasma sample.

As shown in Table 1, the proportion of samples positive for *hMLH1* methylation increases from 12% (16 of 138) of plasma DNA samples before chemotherapy to 33% (45 of 138) at relapse, a significant ($P < 0.001$) increase in *hMLH1* methylation. Twenty five percent (34 of 138) showed *hMLH1* methylation in the relapse sample but not in the prechemotherapy sample and hence evidence of acquisition of *hMLH1* methylation after chemotherapy, consistent with selection for loss of DNA MMR and hence loss of MMR-dependent apoptotic responses. Although 11 of 16 plasma samples with methylated MLH1 prechemotherapy retain methylation at relapse, 5 show apparent loss of methylation. This is most likely to be due to a proportion of patients not having detectable tumor DNA in plasma at relapse and is consistent with the 73% sensitivity of detecting the same genetic change in plasma DNA as present in

Table 1 Plasma DNAs with *hMLH1* methylation and microsatellite instability phenotype

	hMLH1 methylation		Microsatellite phenotype		
	Negative	Positive	MS-S[a]	MSI-L	MSI-H
Pre-chemo	122(88%)	16(12%)	72(84%)	12(14%)	2(2%)
Relapse	93(67%)	45(33%)	48(56%)	27(31%)	11(13%)

[a] MS-S, microsatellite stable; MSI-L, low microsatellite instability (a shift in one locus of at least five loci examined); MSI-H, high microsatellite instability (a shift in more than one locus); Pre-chemo, before chemotherapy.

the tumor reported previously for ovarian cancer at presentation (8). The observation that three of the relapse plasma samples that had apparently lost MLH1 methylation had very low DNA yields (<100 ng/ml plasma) further supports this interpretation.

Because surgical intervention at the time of the relapse is rare, we are unable to compare the changes we observe in plasma DNA with relapse tumor DNA. We have been able to obtain fixed tumor samples taken at presentation for 12 of 138 of these patients. Unfortunately, only two of these show *hMLH1* methylation in plasma DNA, but we do detect *hMLH1* methylation in DNA isolated from the fixed tumor material from these two patients (data not shown). As an alternative approach to validating the detection of methylated *hMLH1* in plasma, we have examined whether there is an association between *hMLH1* methylation and MSI in plasma DNA.

Association between MSI and *hMLH1* Methylation in Plasma DNA. Loss of MMR due to *hMLH1* methylation is associated with a mutator phenotype and acquisition of MSI (20). As a validation of loss of *hMLH1* expression and MMR function in these patient samples, we have examined MSI in plasma DNA from the same patients. DNA was isolated from 96 matched plasma samples from the same patients prechemotherapy or at relapse, as well as from PBMCs, and examined for allelic shifts at six microsatellite loci. Ten prechemotherapy and eight relapse samples were unsuitable for MSI analysis because <5 loci gave sufficient PCR product in one of the plasma DNA samples (86 paired samples were available for analysis). To reduce the probability of false positives, 30 PCR cycles were used for all samples, and products of <30 fluorescent units were excluded from the analysis. Allelic shifts were defined as the appearance of a new allele that is altered in size compared with the PBMC alleles by at least 2 bases (this cutoff was used to avoid confusion with so-called PCR stutter fragments produced during the PCR reaction). Examples of allelic shifts are shown in Fig. 2. MSI was defined as either a shift in one locus of at least five loci examined [low MSI (MSI-L)] or a shift in more than one locus [high MSI (MSI-H)]. For the purposes of the present analysis, if no allelic shift was detected in at least five loci, the sample was defined as microsatellite stable (MS-S).

Evidence of MSI, as defined by MSI-H, is observed in plasma DNA from 2% of patients at presentation, but this increases to 13% at relapse (Table 1). Similarly, MSI-L in plasma increases from 14% in patients at presentation to 31% at relapse. The increase in MSI observed in relapse samples compared with prechemotherapy samples is statistically significant ($P < 0.001$). The increase in MSI we observe after chemotherapy is consistent with previous observations made in small retrospective studies of ovarian cancer that there is increased MSI in residual tumor cells surviving chemotherapy (21).

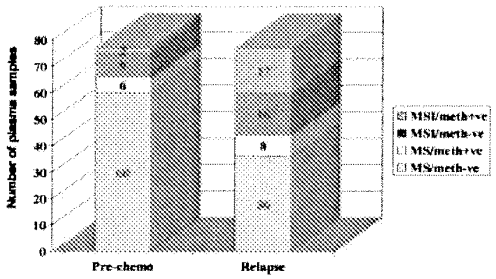

Fig. 3 *hMLH1* methylation and/or microsatellite instability (MSI) phenotype distribution of plasma DNA. Microsatellite-stable (MS-S) samples are those with no allelic shifts in at least five microsatellite loci examined. MSI includes MSI-L and MSI-H. *meth+ve,* samples positive for *hMLH1* methylation by fluorescent methylation-specific PCR; *meth-ve*, samples negative for *hMLH1* methylation by fluorescent methylation-specific PCR.

For 77 of the patient samples, we were able to obtain both MSI and *hMLH1* methylation status in matched samples before chemotherapy and at progression. The proportion of samples with each phenotype is shown in Fig. 3. The percentage of relapse samples that have *hMLH1* methylation has a statistically significant association with MSI-positive status ($P = 0.001$). The proportion of plasma DNA that has methylation of *hMLH1* and is MSI positive (MSI-H and MSI-L) increases from 3% (2 of 77) prechemotherapy to 22% (17 of 77) at relapse. The proportion of MSI-positive DNAs that do not show evidence of *hMLH1* methylation also increases from 12% (9 of 77) prechemotherapy to 21% (16 of 77) at

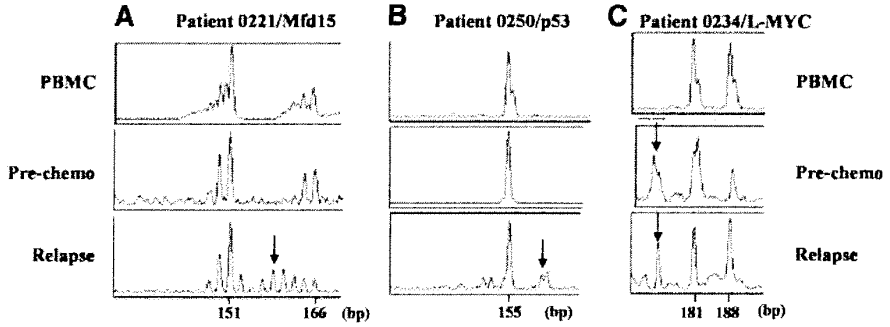

Fig. 2 Examples of allelic shift in plasma DNA. Allele sizes of PCR products at the loci shown were determined in DNA isolated from peripheral blood mononuclear cells (PBMCs), pretreatment plasma (*Pre-chemo*), and relapse plasma (*Relapse*). The sizes (in bp) of the major alleles detected in PBMCs are shown. *Arrows* indicate the presence of new alleles detected in plasma DNA that are not present in PBMC DNA. The microsatellite analyses shown are for (*A*) Mfd15, (*B*) p53, and (*C*) L-Myc.

relapse. Although *hMLH1* methylation is the main mechanism of loss of MMR in sporadic tumors, a proportion of tumors can acquire a MSI phenotype due to alterations in MMR genes other than *hMLH1* methylation (22). A number of plasma DNA samples show methylation of *hMLH1* but no evidence of MSI. It should be noted that only six loci were examined for allelic shifts, and this may be too few to identify instability. However, it is also possible that methylation of *hMLH1* does not cause detectable MSI if only one allele is methylated or inactivation of MMR does not cause a detectable MSI phenotype (for example, inactivation of MMR in cells will only give a detectable MSI phenotype if clonal growth occurs). We have observed ovarian tumors with methylated *hMLH1* that also do not show a detectable MSI phenotype (data not shown). It is possible that methylation of *hMLH1* could reduce MLH1 expression without abolishing MMR activity, and in this context, it is worth noting that reduced expression of MMR proteins can affect drug sensitivity without affecting MSI (23).

Acquired *hMLH1* Methylation and Patient Survival. We have shown that increased *hMLH1* methylation, which correlates with acquisition of a MSI phenotype, is observed in plasma samples at relapse. Next we asked whether this acquired *hMLH1* methylation was associated with patient survival. All laboratory analyses were conducted blinded to clinical outcome. Data from 131 patients were suitable for survival analysis, and of these patients, 78 had died, giving the methylation study 74% power to detect a hazard ratio of 2 for the effect of acquired methylation on survival time from progression. A Kaplan-Meier survival curve of patients whose plasma sample acquires *hMLH1* methylation and those that have not is shown in Fig. 4. Acquisition of *hMLH1* CpG island methylation significantly correlates with poor survival after progression for these epithelial ovarian cancer patients (hazard ratio, 1.83; $P = 0.017$; $n = 131$). Time to progression after primary chemotherapy can influence response to subsequent chemotherapy (24). Indeed, there is a slightly higher proportion of patients who acquire *hMLH1* methylation with a treatment-free interval of <6 months (45% *versus* 39%) and a lower proportion of patients who acquire *hMLH1* methylation with a treatment-free interval of >12 months (16% *versus* 24%) in comparison with patients who do not acquire *hMLH1* methylation. Patient age has also been suggested to influence the propensity for tumors to become methylated (25). However, using multiple Cox regression analysis, the association of acquired *hMLH1* methylation with survival was independent of age and time to progression (hazard ratio, 1.99; $P = 0.007$; $n = 131$).

Any link between acquisition of methylation and histopathological subtype of ovarian cancer could influence these clinical data. The majority of tumors were either serous cystandenocarcinoma or papillary (adeno)carcinoma [38% (50 of 131) and 18% (24 of 131), respectively]. We have not examined each subtype individually because such an analysis would result in small subgroup analysis and greatly weaken the power of the statistical analysis. However, there were 11 clear cell carcinomas and 2 mucinous cystadenocarcinomas, subtypes that have been reported to be associated with particularly poor prognosis in ovarian cancer (26, 27). After adjusting the results of the Cox regression analysis to stratify for these subtypes, a significant association of acquired *hMLH1* methylation with survival was still seen, suggesting that the effect on survival is independent of these ovarian tumor subtypes.

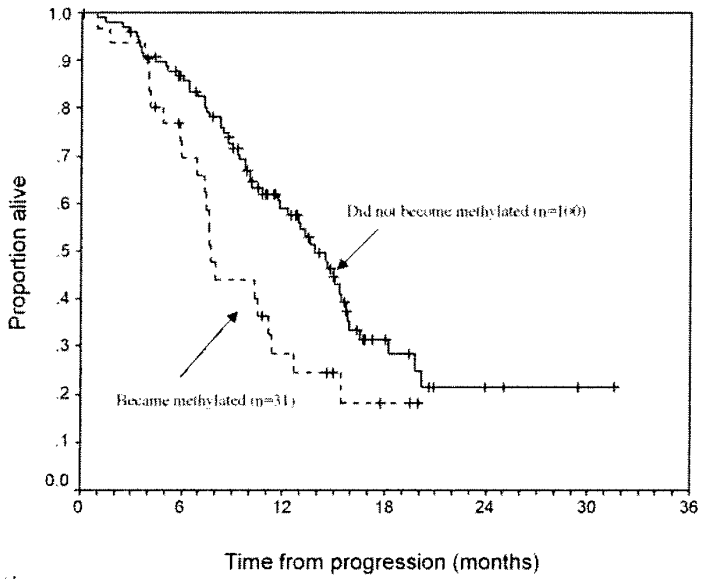

Fig. 4 Kaplan-Meier curves of survival after progression/relapse.

DISCUSSION

The increased *hMLH1* methylation we observe in plasma samples at relapse after carboplatin/taxoid chemotherapy of epithelial ovarian cancer patients is consistent with *in vitro* observations in ovarian cell line models that cisplatin and carboplatin select for loss of an apoptotic response and acquisition of drug resistance, which is associated with loss of expression of MMR proteins and methylation of *hMLH1* (2, 11). Expression of MLH1 prechemotherapy in ovarian cancer does not appear to be associated with survival (28), although previous small studies have demonstrated acquisition of *hMLH1* methylation and loss of MLH1 protein expression after chemotherapy in ovarian cancer (2). The association of acquired *hMLH1* methylation with patient survival we observe is also consistent with a small retrospective study in breast cancer that associated reduced expression of MLH1 protein in matched patient samples taken after neoadjuvant chemotherapy, but not before chemotherapy, with survival (29). It has been argued that because MMR proteins can recognize and bind to platinum cross-links in DNA, this is necessary for MMR-dependent engagement of DNA damage responses such as activation of p53, p73, and other downstream apoptosis signaling pathways (30, 31). Hence, loss of MLH1 expression may lead to reduced engagement of apoptosis either due to reduced cycles of futile repair (32) or reduced stalling (or increased bypass) of lesions in DNA during DNA replication (12).

It is possible that other genes potentially involved in drug sensitivity may also become methylated at relapse. We have shown previously in advanced ovarian cancer that many hundreds of genes can become aberrantly methylated (33). Thus far, we have not examined methylation of other genes in the plasma DNA samples because examination of MMR status by *hMLH1* methylation and MSI was the stated prospective objective, and the study was powered accordingly for statistical analysis. Nevertheless, concordant methylation of genes in ovarian cancer has been observed previously (17), and future analysis of acquired methylation of genes implicated in drug resistance and MMR-dependent apoptosis will be of interest.

The data presented help to validate methylation of *hMLH1* and loss of DNA MMR as clinically relevant mechanisms of acquired drug resistance in epithelial ovarian cancer. A variety of novel epigenetic therapies capable of reversing epigenetic transcriptional silencing are currently undergoing clinical trial, both alone and in combination with cytotoxic therapies. A concern that has been raised regarding such epigenetic therapies is that they may confer resistance to cytotoxic agents such as carboplatin in some tumors (14). However, it should be noted that data presented thus far show sensitization or no effect induced by epigenetic therapies such as DNA methyltransferase inhibitors on ovarian tumor cells *in vitro* or *in vivo*, rather than induced resistance (13). Nevertheless, the data presented here open the possibility of using a relatively noninvasive blood test to stratify and identify those patients who relapse after standard first-line chemotherapy who may benefit most from novel epigenetic therapies alone or in combination with conventional chemotherapy.

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The Disposition and Pharmacokinetics in Humans of 5-Azacytidine Administered Intravenously as a Bolus or by Continuous Infusion[1]

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SUMMARY

The disposition of 5-[4-^{14}C]azacytidine, administered i.v. as a bolus or continuous infusion, was studied in cancer patients. After bolus, plasma ^{14}C levels exhibited a multiphasic disappearance pattern; half-life ($t_{1/2}$, β phase) = 3.4 to 6.2 hr. Of ^{14}C in plasma, <2% was associated with 5-[4-^{14}C]azacytidine 30 min after dose. The ratios of ^{14}C levels were: red cells/plasma, ~0.8; leukocytes/plasma, 1.1 to 2.3; nucleic acids/leukocytes, 0.2 to 0.43; sputum/plasma, 0.05 to 0.17. Urinary excretion (3 days) accounted for 73 to 98% of ^{14}C, <1% in feces. The relative concentration of 5-azacytidine in plasma with continuous infusion stayed higher than with bolus; urinary excretion was similar. Fewer side effects were observed with continuous infusion than with bolus. The stability of 5-azacytidine was determined in various media at several temperatures by thin layer chromatography and nuclear magnetic resonance. At 20° in Ringer's lactate (pH 6.2), the $t_{1/2}$ was 94 to 100 hr. Stability increased with lowering of temperature and pH. From our data we conclude that 5-azacytidine should be given by continuous infusion rather than as a bolus.

INTRODUCTION

5-AC,[3] a pyrimidine analog of cytidine (Chart 1) synthesized[4] in 1964 (25), was shown to possess marked antibacterial (7, 24, 29) and cancerostatic properties (29). In mice, 5-AC decreased the number of circulating lymphocytes and mature bone marrow myeloid cells (30). The compound possesses remarkable inhibitory properties against rapidly proliferating tissues including various experimental neoplastic growths (17, 33, 34). 5-AC inhibits the synthesis of DNA (17, 27) and RNA (5, 13). The mechanism of action probably involves phosphorylation,[5] incorporation into newly synthesized nucleic acids followed by fission of the triazine ring (26). It was found that 5-[^{14}C]AC was phosphorylated in all leukemic tissues studied (17). Further, the drug has been shown to exhibit profound antitumor activity against murine L-1210 leukemia *in vitro* (17) and *in vivo* (30), against Walker 256 carcinoma (3), and against acute lymphoblastic leukemia in AK mice (30).

Studies of the phase specificity of 5-AC revealed that it acted predominantly in the S phase of the mitotic cycle (17) and was lethal to L-1210 cells grown in tissue culture (17). Lloyd *et al.* (18) noted that continuous exposure to low doses of 5-AC was more effective than was short exposure to larger doses and that the drug was relatively inactive in L-1210 cells maintained in nonproliferating state. However, there are some suggestions that the drug may not be purely cell cycle-phase specific and may impair cell structures even in the resting state (23).

5-AC has been found to be promising in the treatment of acute leukemia (1, 11, 14, 15, 35). The apparent instability of 5-AC (26) in solution has necessitated administration by rapid i.v. injection. In clinical trials (6, 15, 20, 21, 31, 32, 35, 37), this has resulted in severe nausea and vomiting. Karon *et al.* (15) found that, in children, if the drug was infused over a period of 10 to 15 min or given in divided doses, gastrointestinal toxicity was reduced. Moertel *et al.* (21) found that, by dividing the dose, less nausea and vomiting occurred.

Thus, the *in vitro* data suggesting that continuous exposure was more effective and the clinical data indicating that intolerable gastrointestinal toxicity was a frequent side effect with bolus doses prompted us to reexplore the stability of 5-AC in solution. Furthermore, we compared the pharmacokinetic data obtained with continuous infusion and single injections using 5-[^{14}C]AC in patients with metastatic cancer and leukemia.

MATERIALS AND METHODS

Patients, whose prior written informed consent was obtained, were selected for study on the basis of metastatic cancer or leukemia, life expectancy of at least 6 weeks, normal bone marrow and blood counts (except for leukemic patients), normal renal function, stable hepatic function, and no other existing disorder. Liver function tests included serum glutamate-oxalacetate transaminase, alkaline phosphatase, prothrombin time, and serum protein. Renal func-

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[3] The abbreviations used are: 5-AC, 5-azacytidine (4-amino-1-β-D-ribofuranosyl-s-triazin-2-(1*H*)-one; NSC- 102816); 5-[^{14}C]AC, 5-[4-^{14}C]azacytidine; TLC, thin-layer chromatography; NMR, nuclear magnetic resonance.

[4] Cultures of *Streptoverticillium ladakanus* can produce 5-AC (2, 9).

[5] The phosphorylation of 5-AC is catalyzed by uridine-cytidine kinase and is blocked by cytidine and uridine (16).

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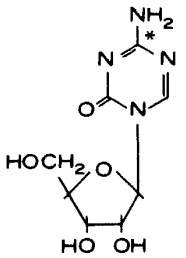

Chart 1. 5-AC (NSC-102816).*, position of ^{14}C.

tion tests included blood urea nitrogen and creatinine clearance. The details of the patients and the doses administered (mg/sq m and μCi) are given in Table 1. After admission, each patient was observed during a control period of 1 to 6 days. This was followed either by a treatment period of 1 day (for single bolus injection studies) or 6 days (for continuous infusion studies) and a follow-up period of 1 to 3 days. The diet was normal except that the patients were fasted from midnight until the morning of the treatment period.

The single i.v. dose of 5-[^{14}C]AC was given at 8:00 a.m. For continuous infusion, the dose of 5-[^{14}C]AC was started at 8:00 a.m. and continued over a 12-hr period. This treatment was then followed with 5-AC every 12 hr for 9 doses; other medications were allowed after the 1st 12-hr period. After a single i.v. dose, 10-ml samples of heparinized blood (with 1000 units of bovine lung heparin; Upjohn Co., Kalamazoo, Mich.) were obtained at predetermined intervals and immediately chilled in ice. Plasma was separated by centrifugation (500 × *g*, 30 min, 0–4°). Urine collections were made every 4 hr on the 1st day and every 24 hr on the 2nd and 3rd day. Daily collections of feces were made for several days. Vomitus, if any, was collected; specimens of sputum were obtained at specified times. In 1 case, leukocytes were also isolated (see below). With the continuous infusion protocol, 20-ml blood samples (heparinized) were collected at various time intervals after the beginning of infusion of 5-[^{14}C]AC. Urine collections were made every 2 hr for the 1st 12 hr and then every 3 hr for the next 12 hr; thereafter, 24-hr collections were obtained for the next 3 days. Feces collections were made for 0 to 24 and 24 to 48 hr. In 1 case, a sample of cerebrospinal fluid was also obtained. All biological samples were stored below 0° and analyzed as soon as possible.

Dose Formulation

5-AC was obtained in a dosage form consisting either of *(a)* 50 mg of the drug and 100 mg of polyvinylpyrrolidone (plasdone-C-15), or *(b)* a 1:1 mixture of the drug with mannitol for i.v. use (Division of Cancer Treatment, National Cancer Institute, NIH, Bethesda, Md.). The labeled compound (5-[^{14}C]AC)[6] was dissolved in distilled water, and the solution was sterilized by passing through a disposable sterile filter (Swinnex 0.22 μm; Millipore Corp., Bedford, Mass.) and added to carrier 5-AC dissolved in sterile distilled water. This final solution was either injected immediately (in 8 to 10 min) as a bolus or added to 500 to 1000 ml of Ringer's lactate (Hartmann's McGaw Laboratories, Glendale, Calif.) for continuous infusion. The 1st 100 ml of solution were injected over a 20-min period and the remainder in the next 11 hr and 40 min.

An aliquot of the dose solution was tested in rats for the absence of pyrogens (19). Male Wistar rats (197 to 217 g, 4 in each group) were given s.c. injections (20 ml/kg) of either: Group A, sterile distilled water; Group B, a 20% suspension of brewers' yeast in sterile water; or Group C, 1.4×10^{-3} M 5-[^{14}C]AC (0.3 μCi/animal) in sterile water passed through a sterile Swinnex filter. Rectal temperatures were measured by a thermocouple at 0, 4, and 24 hr after injections. The average temperatures for Groups A, B, and C for 0, 4, and 24 hr were as follows: Group A, 37.1°, 37.5°, 36.9°; Group B, 36.6°, 38.1°, 37.6°; and Group C, 37.1°, 37.8°, 36.9°. The stability of the solution of 5-AC in Ringer's lactate was determined prior to infusion (see below).

TLC

TLC was carried out on: *(a)* Silica Gel G-coated glass plates without fluorescent indicator (Analtech Uniplate, Newark, Del.), and *(b)* Silca Gel G-coated plastic sheets with fluorescent indicator (Eastman Chromagram 6060). The plates were developed in System 1 (1-butanol:ethanol:water, 49:11:19, v/v/v) at room temperature. Radiochromatography was carried out on glass plates and 1-cm segments of silica gel were scraped and transferred into counting vials; 0.5 ml of methanol was added prior to counting fluid. 5-AC and related compounds (1 μl of 3.5 mM aqueous solutions) were spotted on fluorescent plates (6060) and, after development, the spots were visualized by quenching of fluorescence under UV light (254 nm). The R_F values for 5-AC, 5-azacytosine, and 5-azauracil were 0.35, 0.24, and 0.22, respectively.

Measurement of Radioactivity in Biological Materials and Extracts

Aliquots (up to 2 ml) of plasma or urine were mixed with 18 ml of counting fluid (prepared by mixing 7 g of PPO, 0.36 g of POPOP, 200 ml of Beckman Biosolv BBS-3, and 1 liter of toluene). They were counted alone or in the presence of known amounts of 5-[^{14}C]AC in a Beckman LS-255 liquid scintillation spectrometer (Beckman Instruments, Inc., Fullerton, Calif.) (counting efficiency, 90%). Similarly, ^{14}C was measured in vomitus, sputum, WBC, and spinal fluid; for RBC, 0.3-ml aliquots were oxidized in a Beckman-Harvey combustion instrument, and the resulting $^{14}CO_2$ was trapped in "Harvey carbon-14 cocktail" (R. J. Harvey Instrument Corp., Hillsdale, N.J.) (15 ml; counting efficiency, 70 to 75%). Recoveries (85 to 90%) of ^{14}C after combustion were determined by oxidizing known amounts of 5-[^{14}C]AC added to sucrose. Feces were homogenized with 100 to 500 ml of 95% ethanol. Ten-ml aliquots were centrifuged and 1 to 2 ml of the clear supernatant were counted. This procedure was found to be satisfactory since practically no radioactivity was detected in the residue upon oxidation.

[6] 5-[^{14}C]AC (Lot No. 219-3C, 49.1 mCi/mmole) was synthesized by Monsanto Research Corp., Dayton, Ohio, and was supplied by the Drug Research and Development Chemotherapy Branch, National Cancer Institute, NIH, Bethesda, Md. The radiochemical purity (>95%) was shown by the manufacturer by descending paper chromatography in 1-butanol saturated with water (R_F 0.22). The labeled material as well as carrier were stored at −20°.

Table 1
Description of patients and doses of 5-[^{14}C]AC

Patient[a]	Age (yr)	Wt (kg)	Surface area (sq m)	Dose (mg/sq m)	Diagnosis
1	44	73	1.86	150	Mesothelioma
2	49	103	2.27	200	Adenocarcinoma of lung
3	44	66	1.70	200	Adenocarcinoma of lung
4	27	56	1.67	250	Acute myeloblastic leukemia
5	21	67	1.90	150	Melanoma
6	50	59	1.80	25	Squamous cell carcinoma of lung
7	64	46	1.50	50	Acute myeloblastic leukemia
8	24	52	1.50	75	Adenocarcinoma of colon

[a] Patients 1 to 5 received 5-[^{14}C]AC as a single i.v. bolus; the total dose of 5-AC per week was twice the indicated value. For Patients 6 to 8, who received 5-[^{14}C]AC by continuous infusion over a 12-hr period, the 24-hr dose of 5-AC was twice the indicated value; the total dose of 5-AC administered in 1 week was 10 times the 12-hr dose. Patients 1 and 2 received 25 μCi; all others were given 50 μCi of the labeled drug. Patients 3, 4, and 8 were females. The creatinine clearances in patients 1 to 5 and 7 were 80, 124, 119, 90, 73, and 101 ml/min, respectively.

Measurement of 5-[^{14}C]AC in Plasma

Fresh plasma (3 ml) obtained from patients receiving 5-[^{14}C]AC was diluted with 5 ml of 10^{-2} M Tris buffer, pH 6.5. Then, 3 drops of 10% w/v oxalic acid were added (final pH, 6 to 6.5). After shaking, the mixtures were immediately shell frozen and lyophilized (VirTis Co., Gardiner, N.Y.). The residue was triturated with 10 ml of methanol, sonically extracted for 5 min, and centrifuged for 10 min at room temperature. The precipitate was reextracted with 10 ml of methanol. The final residue was discarded, since essentially no ^{14}C was found in it. The methanol extracts were pooled and evaporated to dryness in a vacuum at room temperature, and the resulting residue was redissolved in 1 ml of methanol. Aliquots of this solution were chromatographed (Silica Gel G-coated plates, without fluorescent indicator; Analtech Uniplate) using System 1.

Isolation of Leukocytes and Nucleic Acids

Leukocytes were isolated by the method of Hirsch (10). Aliquots of blood samples (10 ml) from Patient 4 were mixed with 10 ml of ice-cold 3% w/v dextran (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.; M.W. 400,000) in 0.85% NaCl containing 100 units of heparin per ml. The mixture was kept at 0°, and the RBC were allowed to sediment for 20 to 30 min. The leukocyte-rich suspension was aspirated and centrifuged (10 min, 2000 × *g*, 4°). The resulting pellet was washed with 10 ml of 0.85% NaCl as above. Contaminating RBC were lysed for 10 min at 0° (in 5-ml lyse solution consisting of 1 g sodium citrate dihydrate and 2.5 g NaCl in 1 liter of distilled water; final pH adjusted to 5 with HCl). An equal volume of neutralizing solution (7 g of sodium citrate dihydrate and 15 g of NaCl in 1 liter of distilled water) was added, and the WBC were centrifuged as above and washed twice with 2 ml of 0.85% NaCl solution. The pellet was then suspended in 1 ml of 0.85% NaCl solution and weighed. An aliquot of this suspension was counted and was found to have 98% leukemic blast cells, and another aliquot (0.3 ml) was mixed with 0.3 ml of 0.8 M perchloric acid at 0°. After standing for 10 min the mixture was centrifuged (27,000 × *g* for 10 min at 0°). The supernatant was decanted and aliquots were counted. The precipitate was washed twice with 0.2 M perchloric acid (5 ml) at 0° and resuspended in 1 ml of 0.8 M perchloric acid. After heating (100° for 20 min) and centrifugation, aliquots of the resulting supernatants were counted.

Isolation and Measurement of [^{14}C]Urea from the Urine of a Patient Receiving 5-[^{14}C]AC

To 2 ml of urine (Patient 1) were added 2 ml of an aqueous solution of urea (100 mg/ml) followed by 1.5 ml of 95% ethanol. The mixture was heated (100° for 5 min) and then quickly filtered while hot. The filtrate was cooled to room temperature and again filtered. The remaining precipitate was washed 4 times with 1 ml of hot ethanol. The combined filtrate and washings were evaporated to dryness in a vacuum at 60°. The residue was triturated with hot ethanol and filtered. The filtrate was cooled to −20°, and the resulting crystals of urea were collected by filtration. After 2 recrystallizations from ethanol, urea was dried in a vacuum at 80°. An aliquot of the product from each crystallization was subjected to TLC on Silica Gel G glass plates and developed in System 1. Segments of silica gel (4 mm) were scraped and counted. For visualization, the plates were dried overnight, sprayed with a solution of 0.05% bromcresol green (in 95% ethanol, adjusted to pH 6.5 with dilute NaOH), followed by exposure of the plates to HCl vapors. A blue spot (R_F 0.46) corresponding to urea could be briefly seen against a yellow background.

The presence of ^{14}C associated with urea was specifically determined by treating urea samples isolated from urine with urease and measuring the evolved $^{14}CO_2$ (8). A twin-armed Warburg flask was charged as follows: body, 3 ml of urea solution (1.9 mg/ml) in 5 mM phosphate, pH 6.5, containing 0.5 mM disodium EDTA; 1st side arm, urease (prepared from Jack Bean meal) or water for control; 2nd side

arm, 0.5 ml 1 N HCl; center well, paper wick plus 0.5 ml 1 N NaOH. The flask was tightly stoppered and the contents of the 1st side arm were tipped and mixed with the urea solution. After a 2-hr incubation at 37°, HCl was added to the reaction mixture to release $^{14}CO_2$. After 24 hr, the contents of the central well were transferred into a scintillation vial. The well was washed with several small aliquots of water and the washings were also transferred into the scintillation vial and mixed with counting fluid, and ^{14}C was measured.

Measurement of Partition Coefficient and Binding of 5-[^{14}C]AC

The partition coefficient of 5-[^{14}C]AC was measured at room temperature between M/15 Sörensen buffer, pH 7.4 (28), and various organic solvents (chloroform, *n*-heptane, and peanut oil). An aqueous solution of 5-[^{14}C]AC (10 ml, 10 μg, 0.01 μCi) was shaken with buffer-saturated organic solvents (20 ml) for 40 min. After the phases were separated by centrifugation, ^{14}C was measured in 2 ml of the buffer phase, 1 ml of peanut oil, and 2 ml of chloroform or *n*-heptane (after evaporation).

The binding of 5-[^{14}C]AC to human albumin was measured at 37° and pH 7.4 by 2 methods: *(a)* equilibrium dialysis, and *(b)* molecular sieve *(22)*.

Equilibrium Dialysis. Visking dialysis tubing (VWR Scientific, Atlanta, Ga.; 5/8 inch, size 20) was washed twice and then kept in distilled water for at least 24 hr and blotted dry. In 1 set of experiments, 2 ml of 5% w/v human serum albumin (crystallized, Pentex; Miles Laboratories, Kankakee, Ill.) were placed inside the dialysis bags. These were then placed in test tubes containing 10 ml of Sörensen buffer containing 100 μg, 0.01 μCi of 5-[^{14}C]AC (freshly prepared). In another set of experiments, 200 μg (0.02 μCi) of 5-[^{14}C]AC were added to 2 ml of albumin solution, which was then placed in the dialysis bag and dialyzed in 10 ml of Sörensen buffer. In these experiments, equilibration was achieved by shaking in air (100 cycles/min; Metabolyte Bath, New Brunswick, N. J.) at 37° for 4 hr. Leakage of protein through the dialysis tubing was tested with 40% w/v aqueous trichloroacetic acid. Radioactivity in the inside and outside phases was determined and the binding was calculated.

Molecular Sieve Method. Since 5-AC is not very stable in solutions at 37°, the binding of the drug to human serum albumin (5% w/v in Sörensen buffer, pH 7.4) at 37° was also measured by the molecular sieve method (which consumes much less time). The procedure used was essentially that described by Mu *et al.* (22).

Stability Studies

The stability of 5-AC in various media at several temperatures was studied by 2 methods: *(a)* TLC using 5-[^{14}C]AC, and *(b)* NMR using 5-AC. For Method *a*, the compound was dissolved in water or buffer at various pH's and stored at appropriate temperatures (0–37°) for specific times. Aliquots were analyzed by quantitative TLC (silica gel plates, System 1) as described above. Radioactivity in the zone corresponding to 5-[^{14}C]AC was determined. These values (as percentages) were plotted *versus* time and half-lives ($t_{1/2}$'s) were calculated.

For Method *b*, 5-AC was dissolved in the appropriate medium and NMR studies were carried out at 9–37° on a Bruker Scientific HFX-90 spectrometer (Bruker Scientific, Inc., Elmsford, N. Y.). The spectra were taken at a width of 120 Hz and were time averaged over multiple scans (Nicolet 1074 computer). Tetramethylsilane and benzaldehyde in a 5-mm coaxial capillary served as external references. The rate of decrease (relative to the reference peak) in the height of the C-6 ring proton resonance of 5-AC was taken to be the rate of decomposition. The relative height of resonance peak for C-6 proton was calculated (sample peak height/reference peak height). Multiple regression analysis of the data was performed using 1st-order rate kinetics, and $t_{1/2}$'s at a 95% confidence level were determined.

NMR spectra at 40° were taken on a Varian A-60A spectrometer (Varian Associates, Palo Alto, Calif.). The rate of decrease of the area under the C-6 ring proton resonance of 5-AC (relative to the reference resonance) was assumed to be the rate of decomposition of 5-AC. The $t_{1/2}$'s were determined as described above.

RESULTS

Studies in Patients

Single I.v. Dose. Five patients received 5-[^{14}C]AC as a single bolus dose; plasma levels of ^{14}C (expressed as μg equivalents of 5-[^{14}C]AC per ml of plasma) are shown in Table 2. The data were analyzed by computer, using a program developed by Dr. William Olson (Department of Medicine, Emory University). The plasma ^{14}C levels (C_p, 0 to 12 hr) fit the expression:

$$C_p = Ae^{-k_\alpha t} + Be^{-k_\beta t}$$

where A and B are the intercepts and k_α and k_β are the slopes. The calculated values for A and B, the $t_{1/2}$ of α and β phases, and the apparent volumes of distribution (V_d) are given in Table 2. The $t_{1/2}$ of distribution phase (α) ranged from 16 to 33 min, the $t_{1/2}$ of β phase ranged from 3.4 to 6.2 hr, and V_d ranged from 0.58 to 1.15 liters/kg.

Preliminary studies indicate that the concentration of 5-[^{14}C]AC in plasma declined much faster than total ^{14}C; thus in 1 patient (Patient 1), at 5, 10, 15, and 20 min after dose, the levels of 5-[^{14}C]AC were 40, 37, 27, and 7%, respectively, of the total ^{14}C in plasma. After 30 min the values for 5-[^{14}C]AC were <2% of ^{14}C in plasma. At least 2 metabolites and/or decomposition products of 5-AC were found in plasma by TLC.

The ratio of ^{14}C levels in RBC to plasma was about 0.8 (RBC/plasma ^{14}C concentration ratios for Patient 3 at 5, 15, and 30 min and 1 hr were 0.81, 0.82, 0.85, and 0.80, respectively). The ratio of concentration of ^{14}C in leukocytes to plasma ranged from 1.1 to 2.3, while ^{14}C associated with nucleic acids of WBC was 20 to 43% of ^{14}C in leukocyte (Table 3).

The level of ^{14}C secreted into the sputum was from 5 to 17% of plasma concentrations; the vomitus contained <0.1% of the dose (Table 2). The patients vomited about 6 times (range, 2 to 11) starting 1 hr after the dose (until 14 hr in some cases). The vomitus varied in color from light yellow

Table 2

Plasma levels of ^{14}C in patients after administration of 5-[^{14}C]AC

	Concentration of ^{14}C (5-AC equivalent, μg/ml)							
	After single i.v. dose[b]					During and after continuous i.v. infusion		
Time[a]	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8
5 min	11.9	10.6	15.7	10.2	13.9			
10 min	9.3	9.7						
15 min	8.9	9.3	12.1			0.23		0.32
30 min	8.0	8.6	9.8	7.5	8.5	0.22		0.38
45 min	7.0	7.8	8.0					
1 hr	6.5	7.2	7.1	6.3	6.1	0.22	0.45	0.44
2 hr	4.6	6.1	5.0	5.2	4.4	0.28	0.39	0.79
3 hr	3.7	5.1	4.2	4.7				
4 hr	2.8	4.3	3.8	3.9	3.0	0.39	0.39	0.78
5 hr	2.5	3.8						
6 hr	2.2	3.2	2.3	2.8	2.0	0.38	0.50	0.76
8 hr			1.5		1.2	0.44	0.44	0.62
12 hr	1.0	1.7	0.7	1.7	0.61	0.61[c]	0.93[c]	1.33[c]
16 hr			0.4	1.2				
20 hr			0.2					
24 hr	0.44	0.44	0.11	0.89	0.07	0.13	0.14	0.12
32 hr			0.11					
48 hr		0.46	0.2	0.61				

[a] For single i.v. dose, the time indicated is after drug administration; for continuous infusion, it is from start of infusion. For doses, see Table 1.

[b] Computer analysis of the 0- to 12-hr data of Patients 1 to 5 fits a 2-compartment model ($r > 0.95$). The $t_{1/2}$'s of distribution phase (α) for these patients were: Patient 1, 33 min; Patient 2, 28 min; Patient 3, 16 min; Patient 4, 22 min; and Patient 5, 17 min. The $t_{1/2}$'s for β phase were: 4.7, 5.5, 3.4, 6.2, and 3.5 hr, respectively. The corresponding apparent volumes of distribution were 0.62, 0.58, 0.60, 1.15, and 0.58 liters/kg, respectively. The vomitus contained 0.01, 0.09, 0.03, and 0.02% of dose of ^{14}C in Patients 1, 2, 3, and 4, respectively; ^{14}C (5-AC equivalent) in the sputum obtained at the indicated time was as follows: Patient 1, 1.5 hr, 0.27 μg/ml; Patient 2, 0.5 hr, 0.8 μg/ml, and 1 hr, 0.55 μg/ml; and Patient 3 1 hr, 1.22 μg/ml.

[c] Infusion stopped.

Table 3

Distribution of ^{14}C in leukocytes[a] and nucleic acids isolated from the blood of a leukemic patient (Patient 4) after administration of 5-[^{14}C]AC

	^{14}C (5-AC equivalent) levels		^{14}C in nucleic acids as % of ^{14}C in leukocytes
Time after dose (hr)	Plasma (μg/ml)	Leukocytes (μg/g)	
1	6.3	7.0	27
2	5.2	6.6	31
4	3.9	4.3	20
6	2.8	4.0	30
24	0.9	2.1	43

[a] 98% leukemic blast cells.

Table 4

Cumulative urinary excretion of ^{14}C in patients administered 5-[^{14}C]AC as single i.v. dose[a]

	% of dose excreted in				
Time (hr)	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
0–4	44	38	44	22	47
0–8	67	58	73	50	60
0–12	78	71	82	58	66
0–24	90	85	91	69	69
0–48	96	93	94	77	71
0–72	98	95	95	80	73

[a] Percentage of dose of ^{14}C excreted in feces was as follows: Patient 1, 0.01 (0 to 2 days); Patient 2, 0.36 (0 to 1 day); Patient 3, 0.98 (0 to 2 days); and Patient 4, <0.01 (0 to 2 days). The renal clearance (ml/min) of ^{14}C (5-[^{14}C]AC and metabolites) for 0- to 4-, 4- to 8-, and 8- to 12-hr intervals were as follows: Patient 1, 107, 128, and 210; Patient 2, 115, 118, and 160; Patient 3, 125, 178, and 127; Patient 4, 74, 175, and 93; Patient 5, 127, 77, and 79.

to green; pH ranged from 7.5 to 7.9. Nausea was pronounced and was partially controlled by chlorpromazine or prochlorperazine.

Most of the administered ^{14}C appeared in urine: 69 to 91% and 73 to 98% in 1 and 3 days, respectively, while less than 1% of the dose was present in the feces (Table 4). The calculated amount of ^{14}C remaining in the body showed considerable individual variations in these patients; in 1 case about 27% of the dose was apparently present even after 3 days.

The renal clearance of ^{14}C (drug + metabolite) for the 1st 12 hr after bolus dose varied from 74 to 210 ml/min (Table 4).

Urea was isolated from the urine of Patient 1 and was crystallized twice from 95% ethanol. By TLC, this fraction was shown to be contaminated with materials cocrystallized with urea. [^{14}C]Urea was quantitatively measured by converting it to $^{14}CO_2$ by urease. Of the excreted ^{14}C in 0- to 4-, 4- to 8- to 12-, and 12- to 24-hr urines, 0.8, 1.6, 2.9, and 5.7%, respectively, was associated with urea.

Continuous Infusion. Three patients received 5-[^{14}C]AC

by continuous infusion. Plasma levels and urinary excretion of ^{14}C are presented in Tables 2 and 5. In 1 case (Patient 7, at 6 hr after beginning of infusion) the spinal fluid to plasma concentration ratio of ^{14}C was 0.08. At 15 and 30 min and 1, 2, 4, and 6 hr after beginning of infusion, plasma 5-[^{14}C]AC levels were 70, 46, 42, 34, 15, and 13%, respectively, of the total ^{14}C concentration in plasma. Urinary excretion of ^{14}C (0 to 24 hr) amounted to 85, 83, and 94% of the dose (Table 5) in the 3 patients, respectively.

Partition Coefficient and Binding of 5-[^{14}C]AC

The partition coefficient of 5-[^{14}C]AC (concentration in the organic phase/concentration in the aqueous phase) was found to be <0.005 for all 3 systems studied.

The binding of 5-[^{14}C]AC to 5% w/v human albumin in Sörensen buffer at pH 7.4, 37°, was found to be <1% both by equilibrium dialysis and molecular sieve procedures.

Stability of 5-AC in Solutions

Freshly prepared solutions of 5-[^{14}C]AC were incubated in various media at several temperatures and analyzed by TLC. For NMR the solutions of 5-AC were allowed to remain in the probe for the duration of the experiment.

TLC Method. Almost all of the spotted radioactivity at time 0 was found to be in the zone corresponding to 5-AC. This value was assumed to be 100%. The radioactivity (as percentage of control) associated with zones corresponding to 5-[^{14}C]AC was measured at 1/2, 1, 2, 4, 6, 24, and 48 hr after incubations at 0°, 25°, and 37° in various media. The decomposition of 5-[^{14}C]AC followed 1st-order kinetics; the $t_{1/2}$'s of decay are given in Table 6. 5-AC was more stable in Tris

Table 5
Cumulative urinary excretion of ^{14}C in patients during and after continuous infusion of 5-[^{14}C]AC

Time[a] (hr)	% of dose infused	% of dose excreted in[b]		
		Patient 6	Patient 7	Patient 8
0–2	23	3.1	5.0	21.8
0–4	39	10.9	11.0	36.6
0–6	54	20.8	13.0	42.4
0–8	69	30.2	27.6	42.7
0–12	100	50.7	46.4	51.6
0–24		85.4	82.7	93.5
0–48			91.7	97.2
0–72			94.7	98.4
0–96			96.2	99.0
0–120			96.9	99.5

[a] Time from beginning of infusion (lasting 12 hr).
[b] The renal clearances (ml/min) of ^{14}C (5-[^{14}C]AC plus metabolites) for 0- to 4- and 4- to 8-hr intervals were as follows: Patient 6, 73 and 96; Patient 7, 88 and 103; Patient 8, 84 and 92, respectively.

Table 6
Stability studies of 5-AC

Medium[a]	Method	Temperature	pH	$t_{1/2}$ of decomposition of 5-AC (hr)[b]	No. of points
Distilled water	NMR	40°	6.5	4.4 ± 0.6[c]	15
EDTA (10^{-5} M)/N_2[d]	NMR	40	6.3	8.5 ± 2.0[c]	19
Tris buffer (10^{-2} M)-EDTA (10^{-5} M)	NMR	40	6.3	6.3 ± 1.2	11
Ringer's lactate	NMR	40	6.2	5.6 ± 1.3[c]	13
Ringer's lactate-EDTA (10^{-5} M)/N_2	NMR	40	6.2	5.8 ± 1.0	9
Human plasma (fresh)[f]	NMR	37	7.4[g]	5.2 ± 0.8	14
Human plasma (fresh)[f]	TLC	37	7.4[g]	1.4	5
Human plasma (stored)[f]	NMR	37	7.4[g]	5.4 ± 0.8	12
Human plasma (stored)[f]	TLC	37	7.4[g]	2.0	5
Sörensen buffer (M/15)	TLC	37	7.4	5.0	5
Sörensen buffer (M/15)	TLC	25	7.4	17	5
Tris buffer (10^{-2} M)	TLC	25	6.3	68	6
Ringer's lactate	TLC	25	6.2	65	7
Human plasma (fresh)[f]	TLC	25	7.4[g]	7.0	5
Human urine	TLC	25	6.5[g]	70	7
Ringer's lactate	NMR	20	6.2	94 ± 5	32
Ringer's lactate	NMR	20	6.2	100 ± 6	25
Ringer's lactate	NMR	9	6.2	170 ± 7	74
Tris buffer (10^{-2} M)	TLC	0	6.3	528	7
Human plasma (fresh)[f]	TLC	0	7.4[g]	53	7
Human urine	TLC	0	6.5[g]	288	7

[a] Concentration of 5-AC = 30 mM (NMR) and 5-[^{14}C]AC = 3.5 mM (TLC).
[b] Each $t_{1/2}$ determination represents a single experiment. In the NMR studies, experiments were carried out for 170 to 190 min (0.5 to 2.0 half-lives). For the TLC, incubations were carried out up to 48 hr.
[c] $t_{1/2}$ ± 95% confidence level.
[d] EDTA, aqueous disodium EDTA.
[e] The R^2 values for these 2 determinations were between 0.90 and 0.93; for others, >0.96 (NMR).
[f] Fresh plasma for each experiment was obtained from different individuals. Stored plasma was stored at 0° for 1 to 10 weeks.
[g] pH was not controlled.

buffer (pH 6.3, 10^{-2} M) or Ringer's lactate at pH 6.2 and least stable in fresh heparinized plasma.

NMR Method. The resonance due to the proton at C-6 of 5-AC occurred at 8.7 ppm (singlet) in D_2O solution at 20°; the aldehyde proton of benzaldehyde resonated at 9.8 ppm relative to the reference, tetramethylsilane in the capillary. In Ringer's lactate at 20°, the resonances due to C-6 and benzaldehyde protons occurred at 9.0 and 9.9 ppm, respectively. These chemical shifts varied slightly with temperature and the composition of the medium. The decomposition of 5-AC (in D_2O) is accompanied by the progressive disappearance of the resonance at 8.7 ppm and the appearance of a new peak of 8.5 ppm (Chart 2). The addition of formic acid to the partially decomposed solution of 5-AC increased the intensity of the peak at 8.5 ppm. The resonance for C-6 protons of azacytosine or 5-azauracil occurred at 8.2 ppm (in alkaline D_2O solution).

For quantitative purposes 16 to 32 scans were taken with an accumulation time of 27 to 54 min. The molarities of the protons present were assumed to be proportional to the peak heights of the corresponding resonances. Relative peak heights were determined relative to the aldehyde proton resonance of benzaldehyde. Multiple regression analysis of the data was performed using 1st-order kinetics and the $t_{1/2}$'s at a 95% confidence level are shown in Table 6.

DISCUSSION

The disposition of 5-[^{14}C]AC was studied in a limited number of patients. The drug was administered i.v. either as a single bolus dose or as a continuous infusion; a comparison of the 2 modes of administration was 1 of the major objectives of this study.

A clinical evaluation of these 2 modes of therapy has been carried out by the Southeastern Cancer Study Group. In a Phase I study of twice-weekly rapid i.v. injections (35), it was found that all patients had severe gastrointestinal toxicity when a single dose exceeded 100 mg/sq m. When given by continuous i.v. infusion, less gastrointestinal toxicity occurred in doses less than 200 mg/sq m (36). In the present study, similar observations were made. Thus the continuous infusion is a more tolerable method of administration.

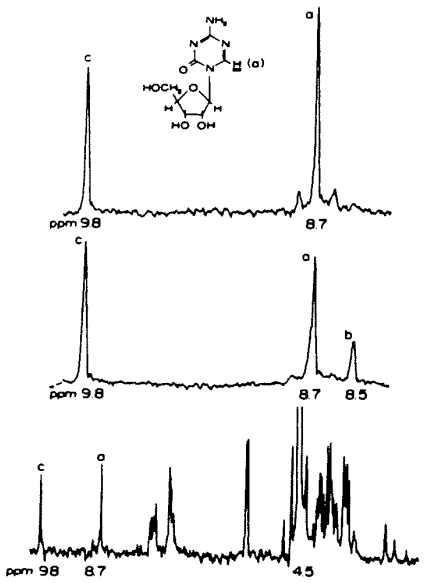

Chart 2. NMR spectrum of 5-AC in D_2O at 20°. *Upper* and *middle panels*, (expanded scale) showing resonance peaks due to *(a)* proton at C-6 of 5-AC (8.7 ppm), *(b)* proton in the decomposition product (8.5 ppm), and *(c)* aldehyde proton in benzaldehyde, used as reference (9.8 ppm). *Lower panel*, survey of 5-AC in D_2O. The resonance at 4.5 ppm is due to the proton in HOD.

The plasma level decline of ^{14}C after the bolus dose of 5-[^{14}C]AC was multiphasic; the pattern was similar to that obtained by Troetel *et al.* (32). The drug distributes rapidly after administration. The apparent volume of distribution of the drug (V_d) agreed with: *(a)* the low organic/aqueous partition coefficient and *(b)* the lack of binding to human albumin. In 1 patient (leukemic) higher V_d may have been due to selective uptake and incorporation of the drug (and/or metabolites) by the leukocytes. The levels of ^{14}C RBC (80% of plasma levels) reflect the lack of binding of 5-AC to plasma proteins; it is also an indication of the absence of binding to the constituents of the red cells.

A small but measurable fraction of the administered radioactivity appeared in the vomitus, suggesting gastric and/or biliary secretion of the drug (and/or metabolites). The salivary ^{14}C levels were about 10% of the plasma levels. The spinal fluid to the plasma concentration ratio of ^{14}C was similar to that reported previously (32).

The concentration of 5-[^{14}C]AC in plasma was determined in a preliminary fashion. At least 2 metabolites and/or decomposition products of 5-AC were found in plasma. The initial plasma level pattern of 5-AC after a single bolus injection reflects at least 3 processes: *(a)* the distribution phase, *(b)* metabolism, and *(c)* nonenzymatic degradation. At 30 min after the administration of the drug, plasma levels of ^{14}C were 70% of the concentration at 5 min. However, ^{14}C associated with the parent drug was <2% of the total, indicating rapid metabolism and degradation. The maximum nonenzymatic decomposition (both in plasma and tissues) probably accounts for only 20% of the overall decrease of 5-AC in plasma at 30 min (see later).

In patients who received the dose by continuous infusion, plasma ^{14}C levels indicated some accumulation. In contrast to the bolus dose, ^{14}C associated with the parent drug in plasma was much higher; after 6 hr of continuous infusion about 13% of plasma ^{14}C was present as the parent drug.

We sought optimum conditions for *(a)* preparation and storage of solutions of 5-AC for continuous infusion and *(b)* analysis of 5-AC in biological materials. Therefore a study of the stability of the drug was carried out in solution at various pH's, temperatures, and media.

The stability of 5-AC was measured by 2 independent methods: *(a)* TLC, using 5-[^{14}C]AC, and *(b)* NMR, using nonlabeled compound. Studies with TLC were done in a preliminary fashion and the results confirm data obtained from the NMR studies. The stability studies with NMR are more reliable, since we measured the disappearance of the resonance peak corresponding to the proton at C-6 of the pyrimidine moiety and the simultaneous appearance of a new peak. Since the addition of formic acid to this partially decomposed solution of 5-AC resulted in an increase in the intensity of the new peak, it is possible that it is due to formic acid formed from the *N*-formyl intermediate pro-

posed by Pithova *et al.* (26) and not due to decomposition products of 5-AC (5-azacytosine and/or 5-azauracil).

Although our data are not sufficient to propose a mechanism for the decomposition of 5-AC, one may write a scheme for the decomposition similar to that proposed by Pithova *et al.* (26). The *N*-formyl intermediate yields formic acid and 1-β-D-ribofuranosyl-3-guanylurea upon hydrolysis. The formic acid may contribute partially to the toxic and therapeutic effects of 5-AC.

In fresh human plasma at 37°, 5-AC was fairly unstable. The stability increased by lowering of temperature. In distilled water at pH 6.5 and 40°, $t_{1/2}$ was 4.4 hr; the addition of disodium EDTA and bubbling the solution with nitrogen doubled the $t_{1/2}$. It is possible that disodium EDTA chelates ions, *e.g.*, iron (which may still be present at very low concentrations in distilled water), which may act as a catalyst in the decomposition of 5-AC. Bubbling with nitrogen (to remove dissolved oxygen) may also have increased the stability. However, when the same technique (EDTA and N_2) was used with Ringer's lactate, only a small increase in $t_{1/2}$ was observed. Apparently, the small amount of EDTA added to the solution was used up by calcium present in Ringer's lactate.

The temperature and pH studies indicated that decomposition of 5-AC was dependent on both of these parameters. The stability increased with a decrease in pH or temperature.

The stability of 5-AC was determined in Ringer's lactate since the latter is used as a medium for infusion of 5-AC in humans. In Ringer's lactate, 5-AC was fairly stable at 20° (pH 6.2, $t_{1/2}$ = 94 to 100 hr; for 10% decomposition the value was 14 to 15 hr). From these data we have concluded that, if the drug solution is prepared every 8 hr and infused at room temperature, the decomposition would not be more than 10% over the infusion period. The decomposition would be even less if the drug solution could be maintained at a still lower temperature during infusion.

Earlier studies of stability of 5-AC (4, 26) were done either under nonphysiological conditions such as high temperature (50–100°) or at acid or alkaline pH. Cihak and Sorm (4) studied the stability by determining the loss of biological activity of 5-AC (by measuring the inhibition of the growth of *Escherichia coli*). Pithova *et al.* (26) studied the hydrolysis of 5-AC by using paper chromatography and changes in molar absorptivity. The reliability of the latter method is doubtful, since the absorbance (at 255 nm) of the solution of 5-AC increases initially with time and then decreases.

From our studies we conclude that 5-AC should be given to patients by continuous infusion rather than as a single bolus. This manner of administration results in lower incidence of side effects (36); plasma concentration of the parent drug stays higher for a longer period of time.

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Addendum

After submission of this manuscript, we studied the stability of 5-AC in the formulation used for i.v. injection (containing 1:1 mannitol) at room temperature by NMR. The pH of a 0.03 M solution of the injectable drug in Ringer's lactate was 7.1 (for pure 5-AC, to pH was 6.4 under the same conditions). The $t_{1/2}$'s of decomposition of the injectable drug (0.03 M) at pH 7.1 and 6.3 (adjusted with HCl) in Ringer's lactate at 20 ± 1° were 69 ± 3 and 80 ± 4 hr, respectively.

By [^{13}C]NMR it was found that one of the products of the composition of 5-AC in water was formic acid. This is in agreement with the data obtained from the proton-NMR study.

The degradation of 5-AC has recently been studied by a UV method [Notari, R. E., and DeYoung, J. L. Kinetics and Mechanism of Degradation of the Antileukemic Agent 5-Azacytidine in Aqueous Solutions. J. Pharm. Sci., *64:* 1148–1157, 1975].

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REVIEW ARTICLE

MECHANISMS OF DISEASE

Cancer Stem Cells

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THE DEEPENING OF OUR UNDERSTANDING OF NORMAL BIOLOGY HAS MADE it clear that stem cells have a critical role not only in the generation of complex multicellular organisms, but also in the development of tumors. Recent findings support the concept that cells with the properties of stem cells are integral to the development and perpetuation of several forms of human cancer.[1-3] Eradication of the stem-cell compartment of a tumor also may be essential to achieve stable, long-lasting remission, and even a cure, of cancer.[4,5] Advances in our knowledge of the properties of stem cells have made specific targeting and eradication of cancer stem cells a topic of considerable interest. In this article, we discuss the properties of cancer stem cells, outline initial therapeutic strategies against them, and present challenges for the future.

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BACKGROUND AND DEFINITIONS

Stem cells occur in many different somatic tissues and are important participants in their physiology (Fig. 1). Populations of cells that derive from stem cells are organized in a hierarchical fashion, with the stem cell residing at the apex of the developmental pathway (Fig. 2). Stem cells have three distinctive properties: self-renewal (i.e., at cell division, one or both daughter cells retain the same biologic properties as the parent cell), the capability to develop into multiple lineages, and the potential to proliferate extensively. The combination of these three properties makes stem cells unique. The attribute of self-renewal is especially notable, because its subversion is highly relevant to oncogenesis and malignancy.[6,7] Aberrantly increased self-renewal, in combination with the intrinsic growth potential of stem cells, may account for much of what is considered a malignant phenotype.

Many studies performed over the past 30 to 40 years, when viewed collectively, have shown that the characteristics of stem-cell systems, the specific stem-cell properties described above, or both, are relevant to some forms of human cancer.[3,4,8,9] Biologically distinct and relatively rare populations of "tumor-initiating" cells have been identified in cancers of the hematopoietic system, brain, and breast.[10-13] Cells of this type have the capacity for self-renewal, the potential to develop into any cell in the overall tumor population, and the proliferative ability to drive continued expansion of the population of malignant cells. Accordingly, the properties of tumor-initiating cells closely parallel the three features that define normal stem cells. Malignant cells with these functional properties have been termed "cancer stem cells" (Fig. 2).

Given these features, it is possible that cancer stem cells arise by mutation from normal stem cells. However, several lines of evidence indicate that cancer stem cells can also arise from mutated progenitor cells.[14-17] Such progenitors (also known as "transit-amplifying cells") can possess substantial replicative ability, but they do not usually have the self-renewal capacity of stem cells. To become a cancer stem

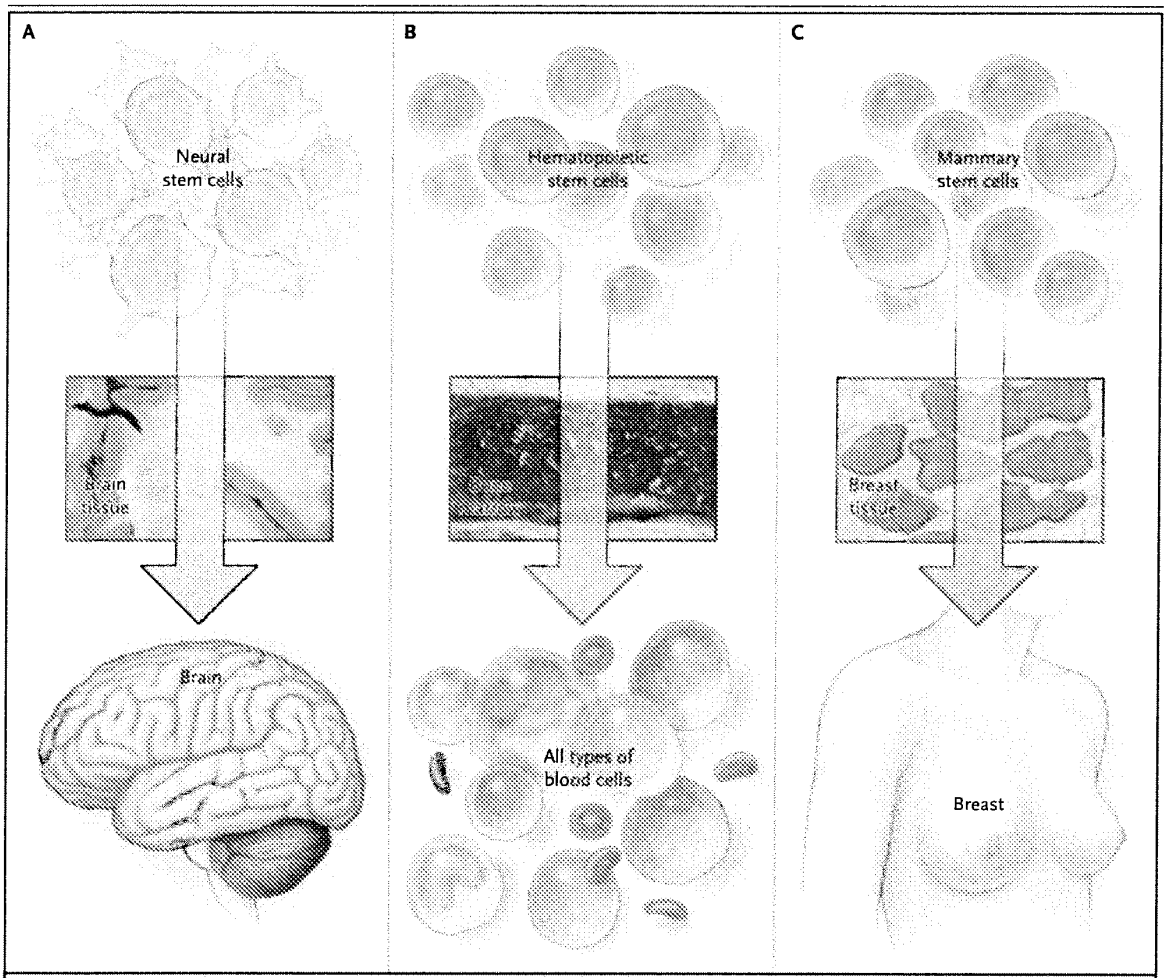

Figure 1. **Examples of Stem Cells Found in Adult Somatic Tissues.**
Neural stem cells generate cells in the central nervous system (Panel A). Hematopoietic stem cells generate mature blood cells (Panel B). Mammary stem cells generate breast tissue (Panel C).

cell, a progenitor cell must acquire mutations that cause it to regain the property of self-renewal. A detailed discussion of the origins of cancer stem cells is beyond the scope of this review, but it is important to acknowledge the possibility that multiple pathways and processes can give rise to cancer stem cells.

Although specific features of normal stem cells may be preserved to greater or lesser degrees in cancer stem cells, the key issue for consideration with regard to tumor biology is that a small subgroup of the cells in a tumor — the cancer stem cells — are essential for its growth. The concept of cancer stem cells can, however, vary in different contexts. For example, cancer stem cells can be the source of all the malignant cells in a primary tumor, they can compose the small reservoir of drug-resistant cells that are responsible for relapse after a chemotherapy-induced remission, or they can give rise to distant metastases (Fig. 3). The biologic features of cancer stem cells in each of these instances may differ, suggesting that the acquisition of features associated with tumor progression, such as genetic instability and drug resistance, will also be associated with cancer stem cells.

It is becoming evident that a cancer treatment that fails to eliminate cancer stem cells may allow

regrowth of the tumor. In cases in which bulk disease is eradicated and chemotherapy is given, only to be followed by a relapse, a plausible explanation is that the cancer stem cells have not been completely destroyed (Fig. 3B). Therapeutic strategies that specifically target cancer stem cells should eradicate tumors more effectively than current treatments and reduce the risk of relapse and metastasis.

CANCER STEM CELLS IN THE HEMATOPOIETIC SYSTEM

The hematopoietic system is the best characterized somatic tissue with respect to stem-cell biology. Over the past several decades, many of the physical, biologic, and developmental features of normal hematopoietic stem cells have been defined[18,19] and useful methods for studying stem cells in almost any context have been established. Hematopoietic-cell cancers such as leukemia are clearly different from solid tumors, but certain aspects of hematopoietic stem-cell biology are relevant to our understanding of the broad principles of cancer stem-cell biology.[6] In various types of leukemia, cancer stem cells have been unequivocally identified, and several biologic properties of these stem cells have been found to have direct implications for therapy.[1,20-22]

Cancer stem cells are readily evident in chronic myelogenous leukemia (CML)[23] and acute myelogenous leukemia (AML),[10,11] and they have been implicated in acute lymphoblastic leukemia (ALL).[24-26] CML stem cells have a well-described stem-cell phenotype and a quiescent cell-cycle status. Similarly, AML stem cells are mostly quiescent,[27-30] suggesting that conventional antiproliferative cytotoxic regimens are unlikely to be effective against them. AML stem cells have surface markers, such as the interleukin-3–receptor α chain, that are not present on normal stem cells.[31] These markers may be useful for antibody-based[32] or other related therapeutic regimens.[33,34] Early efforts have demonstrated the usefulness of antibodies against the CD33 antigen in the treatment of AML,[35,36] and recent reports indicate that CD33 is expressed on some leukemia stem cells.[37] Continued development of immunotherapy against stem-cell–specific antigens is warranted.

There has been extensive research on drugs that specifically modulate pathways implicated in leukemia-cell growth (i.e., "targeted" agents).[38,39]

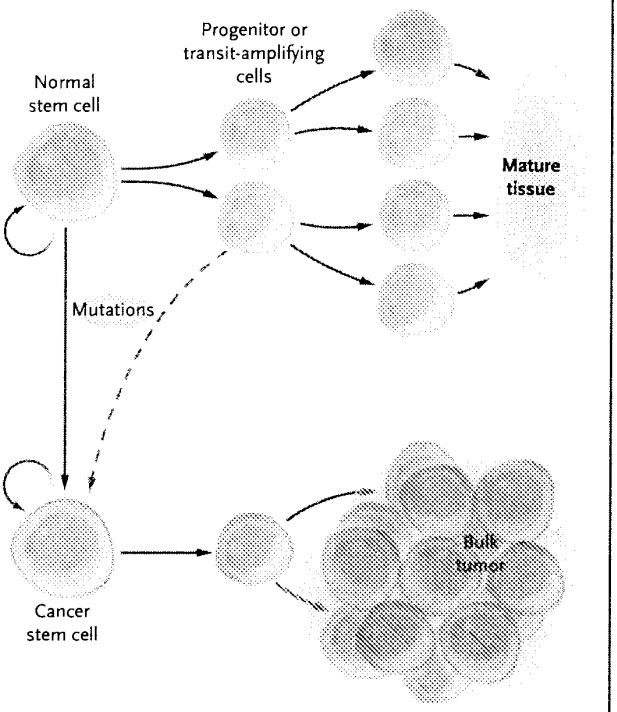

Figure 2. Stem-Cell Systems.

Normal tissues arise from a central stem cell that grows and differentiates to create progenitor and mature cell populations. Key properties of normal stem cells are the ability to self-renew (indicated by curved arrow), multilineage potential (indicated by cells of different colors), and extensive proliferative capacity. Cancer stem cells arise by means of a mutation in normal stem cells or progenitor cells, and subsequently grow and differentiate to create primary tumors (the broken arrow indicates that specific types of progenitors involved in the generation of cancer stem cells are unclear). Like normal stem cells, cancer stem cells can self-renew, give rise to heterogeneous populations of daughter cells, and proliferate extensively.

Use of the ABL kinase inhibitor imatinib mesylate (Gleevec) to treat CML has had particularly interesting results.[40] Despite the remarkable clinical responses achieved with imatinib, however, residual disease persists in many patients. In vitro studies indicate that inhibition of the CML translocation product BCR-ABL is sufficient to eradicate most or all leukemia cells, but the drug does not appear to kill CML stem cells.[41] Imatinib primarily affects the progeny of cancer stem cells, so CML usually recurs when therapy is discontinued.[42] Furthermore, although the newly approved CML agent dasatinib is effective for imatinib-resistant disease, recent data suggest that it too may fail to eradicate CML stem cells.[43]

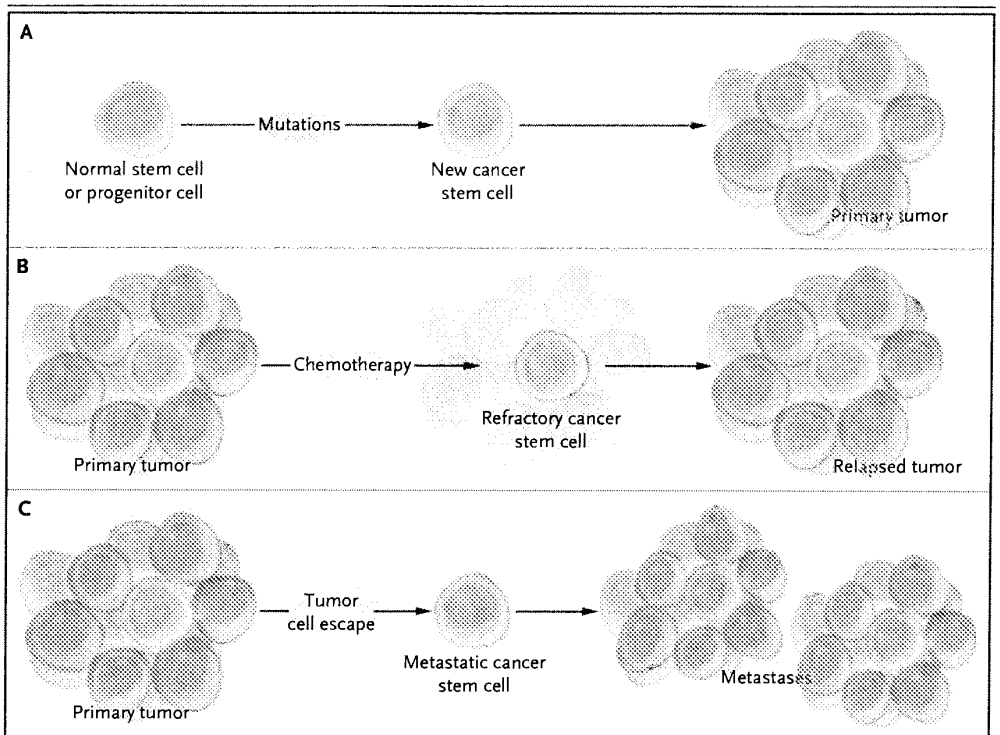

Figure 3. **Scenarios Involving Cancer Stem Cells.**

For tumors in which cancer stem cells play a role, at least three scenarios are possible. First, mutation of a normal stem cell or progenitor cell may create a cancer stem cell, which will then generate a primary tumor (Panel A). Second, during treatment with chemotherapy, the majority of cells in a primary tumor may be destroyed, but if the cancer stem cells are not eradicated, the tumor may regrow and cause a relapse (Panel B). Third, cancer stem cells arising from a primary tumor may emigrate to distal sites and create metastatic lesions (Panel C).

Unique molecular features of leukemia stem cells may provide opportunities for therapeutic intervention. For example, there is evidence of constitutive activation of both the nuclear factor-κB (NF-κB) and phosphatidylinositol 3′ (PI3) kinase signaling pathways in AML stem cells.[28,44] Neither NF-κB nor PI3 kinase activity is detectable in resting, normal hematopoietic stem cells, so both of these molecular factors could be tumor-specific targets. Two studies with different methods of pharmacological inhibition of NF-κB have reported specific eradication of AML stem cells in vitro, without apparent harm to normal hematopoietic stem cells.[45,46] A separate study demonstrated that inhibition of PI3 kinase reduced the growth of AML stem cells.[44] Similarly, inhibition of the downstream PI3-kinase mammalian target of rapamycin (mTOR) appears to enhance the activity of the chemotherapeutic agent etoposide against AML stem cells.[47] Inhibition of mTOR also blocks the growth of leukemia-initiating cells in a mouse model of AML.[48] Taken together, these findings indicate that leukemia stem-cell–specific therapies may be attainable.

CANCER STEM CELLS IN THE CENTRAL NERVOUS SYSTEM

Isolation of cancer stem cells of the central nervous system (CNS) has been achieved by means of antigenic markers and by exploiting in vitro culture conditions developed for normal neural stem cells. As was first observed in 1992,[49,50] CNS cells grown on nonadherent surfaces give rise to balls of cells (neurospheres) that have the capacity for self-renewal and can generate all of the principal cell types of the brain (i.e., neurons, astrocytes, and oligodendrocytes). Neurospheres in which the stem-cell compartment is maintained

can be repeatedly split apart into single cells; a small fraction of these cells can generate a new neurosphere (Fig. 4). This capacity for repeated generation of neurospheres from single cells is generally viewed as evidence of self-renewal.[51,52] More recent studies have demonstrated that normal neural stem cells express a cell-surface protein that can be detected with an antibody against the AC133 (CD133) epitope,[53] a marker commonly found on stem cells and progenitor cells in various tissues.[54]

Application of the strategies used to generate neurospheres to specimens obtained from gliomas[55] or purification of CD133-positive cells from human gliomas[56] allows for the isolation and growth of tumor stem-cell populations. In both cases, the cancer stem-cell population is essential for establishing a tumor in vivo. Transplantation of as few as 100 CD133-positive human glioma cells into the brains of immunodeficient mice initiates the development of a glioma, whereas no tumors result from transplantation of 10^5 CD133-negative cells from the same tumors.[12]

Many studies have demonstrated that the expression of stem-cell–like properties in CNS tumor cells does not necessarily suggest that these cells originated from stem cells. In experimental systems, the expression of cooperating oncogenes in lineage-restricted progenitor cells of the CNS can yield tumors with the cytopathological characteristics of the most malignant CNS tumor (i.e, glioblastoma multiforme). For example, expression of the *ras* and *myc* oncogenes in oligodendrocyte progenitors yields cells that readily form tumors when transplanted in vivo.[57] These studies suggest that a cancer stem cell need not be derived from a bona fide tissue-specific stem cell, but instead can arise from a committed progenitor cell that acquired stem-cell–like properties when it underwent oncogenic transformation.

From a therapeutic perspective, the development of treatments directed against cancer stem cells in the brain is likely to progress substantially during the next several years. The state of knowledge of the stem cells and progenitor cells that build the CNS is sufficiently advanced to permit side-by-side analysis of these populations of cells with CNS tumor cells. Furthermore, a powerful advantage of studies of the CNS is that all of the major precursor (i.e., replicating) populations can be grown as purified populations with the capacity for extended division of stem cells and progenitor cells in vitro.[58-61] Therefore, it should be feasible to conduct high-throughput in vitro analyses to search for compounds that selectively kill cancer stem cells without killing the normal cells of the CNS.

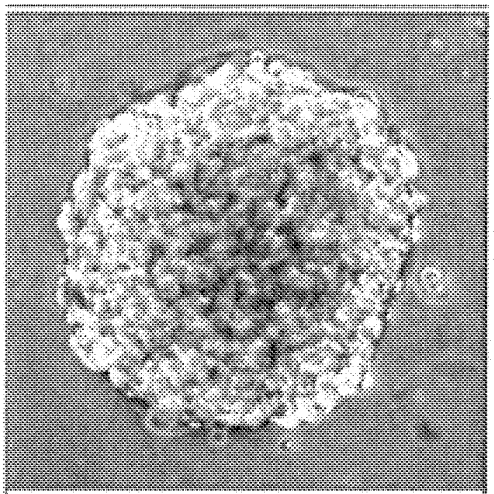

Figure 4. Primary Human Neurosphere.

CANCER STEM CELLS IN THE BREAST

In addition to cancers of the hematopoietic system and the CNS, the third major human cancer in which cancer stem cells have been definitively identified is breast cancer. Studies by Al-Hajj et al. of specimens from patients with advanced stages of metastatic breast cancer demonstrated that cells with a specific cell-surface antigen profile (CD44-positive and CD24-negative) could successfully establish themselves as tumor xenografts.[13] The experiments were conducted with immunodeficient mice, and the cells were transplanted into the mammary fat pad to provide an environment similar to that in human breast cancer. As observed for analogous studies in AML and gliomas, only the relatively rare subgroup of cancer stem cells could successfully propagate the tumor in vivo, whereas the majority of malignant cells failed to recapitulate the tumor. Furthermore, the purified CD44-positive and CD24-negative cells could differentiate and give rise to cells similar to those found in the bulk tumor population.

Definition of the characteristics of both normal cells and cancer stem cells in the breast has advanced rapidly.[62-67] Recent studies have provided detailed characterizations of normal breast

stem cells in mice and have demonstrated the functional potential of such cells by virtue of their ability to completely regenerate a mammary gland when transplanted into a suitable host environment.[68,69] With the experimental tools developed for characterization of normal mammary stem cells, further elucidation of the biologic properties of breast-cancer stem cells should be forthcoming.

CHALLENGES FOR THERAPY TARGETED AGAINST CANCER STEM CELLS

The development of treatments that target cancer stem cells is an important objective, but the challenges are formidable. First, to design treatments that selectively eradicate cancer stem cells, it is useful to have the cognate normal stem cell or progenitor cell. This step requires the development of assays to characterize the function of normal stem cells and the means to define physical features (i.e., cell-surface antigen markers) that will permit their isolation. Without this knowledge, it is impossible to know whether a candidate drug is also cytotoxic to normal stem cells. Second, we need similar ways to describe cancer stem cells and appropriate functional assays must be validated. Third, it is critical to understand how cancer stem cells differ from normal stem cells, particularly with regard to mechanisms controlling cell survival and responses to injury. Ideally, a therapy should target pathways uniquely used by cancer stem cells to resist extrinsic insults or to maintain steady-state viability. Fourth, we must understand how therapies that effectively target the bulk of tumor cells fail to eradicate cancer stem cells. The reasons for this phenomenon may provide important clues for developing more effective and comprehensive regimens to attack both the tumor stem cells and the bulk of the disease.

An additional challenge in targeting cancer stem cells is to understand how the properties of stem cells make them particularly difficult to kill. Leukemia cancer stem cells reside in a largely quiescent state with regard to cell-cycle activity,[27,30] like their normal counterparts. Consequently, typical cytotoxic regimens that target rapidly dividing cells are unlikely to eradicate such cells. Selective targeting will therefore require regimens that kill cells independently of the cell cycle, or that selectively induce cycling of cancer stem cells. Another common feature of stem cells is expression of proteins associated with the efflux of xenobiotic toxins (e.g., multidrug-resistant proteins and related members of the ATP-binding cassette [ABC] transporter family). A variety of cancer cells, particularly during relapse, express such proteins, thus providing resistance to many chemotherapeutic agents.[70-73] The extent to which cancer stem cells can mobilize all of the measures provided by evolutionary history to protect normal stem cells is not yet known, but this information is likely to be biologically and clinically significant.

A further concern is that normal stem cells and progenitor cells may prove to be more sensitive than cancer stem cells to the effects of chemotherapy. Normal colon stem cells, for example, can inhibit DNA repair mechanisms and thereby undergo apoptosis in response to DNA damage; this mechanism avoids the accumulation of harmful mutations.[74] If, however, colon-cancer cells evade this protective mechanism, then chemotherapy could preferentially spare them. Recent studies have demonstrated that normal hematopoietic stem cells undergo premature senescence (i.e., cellular "aging") when exposed to ionizing radiation or busulfan.[75,76] This process impairs the growth and developmental potential of hematopoietic stem cells. If leukemia stem cells fail to undergo senescence, as predicted by recent studies of the genesis of cancer,[77,78] then we would expect that malignant stem cells would actually have a growth advantage after treatment with certain agents. Furthermore, it is plausible that successive cycles of chemotherapy only exacerbate the situation by increasing harm to the normal stem-cell pool (by inducing senescence) and concomitantly increasing the growth advantage of cancer stem cells, which are resistant to senescence. Clearly, a better understanding of normal and tumor stem cells is of great importance not only in designing new therapies, but also in understanding the biologic and clinical consequences of existing regimens.

If a clinical remission is achieved, the presence of residual drug-resistant cancer stem cells can initiate a relapse. Hence, we must develop better methods for detection and quantitation of cancer stem cells in patients receiving cancer therapy. Intriguing findings in leukemia indicate that the level of residual disease directly correlates with the long-term outcome[79,80]: if the number of primitive leukemia cells can be reduced below critical threshold levels, it may not be necessary

to completely eradicate the malignant clone. Whether such residual cells are truly cancer stem cells remains to be determined, but the findings nonetheless suggest that sensitive real-time methods of cancer stem-cell detection are an important priority.

In designing specific regimens for cancer stem cells, several strategies should be considered. Given the likelihood that aberrant regulation of self-renewal is central to cancer stem-cell pathology, targeting pathways that mediate self-renewal is an attractive option. An important unknown factor is the degree to which inhibition of self-renewal mechanisms can be tolerated, because the pathways controlling self-renewal are central to a variety of biologic functions. However, even if the targeting of self-renewal pathways is feasible, we do not know whether it would kill cancer stem cells or simply suppress them. For these reasons, an alternative is to interfere with cancer stem-cell–specific survival pathways. For example, strategies that inhibit survival mechanisms or the oxidative state of the cell may be selectively cytotoxic to leukemia stem cells.[21] Antibody-based or ligand-based therapy also appears to be a promising way to destroy cancer stem cells. A small number of target antigens on cancer stem cells have been described, and with further characterization of purified populations, additional targets are likely to become available. It remains to be determined, however, whether these and other targets will distinguish cancer stem cells from normal tissues.

SUMMARY

There is now abundant evidence that stem-cell properties are highly relevant to the biology of several human cancers. However, many key questions remain. At the most fundamental level, we must determine to what extent stem-cell biology is relevant to all the major forms of human cancer. For this reason, it is premature to overstate the general role of stem cells in cancer. Nonetheless, the eradication of cancer stem cells will be necessary to improve the outcome of treatment for at least some cancers. An interesting question is whether different types of cancer stem cells have the same Achilles' heel; it should be possible to determine whether the same tumor-specific mechanisms of growth and survival are active across multiple cancer types. Because certain features of normal stem cells are conserved in different tissues,[81] determining whether there is similar conservation among cancer stem cells will be useful in the design of new therapies.

Another important issue to investigate is how existing chemotherapy agents affect the evolution of cancer stem cells during conventional treatment regimens. This question relates to both the sensitivity of normal stem cells, as compared with malignant ones, and the mechanisms by which drug resistance may arise. Do current forms of treatment provide a competitive advantage for cancer stem cells, and if so, does that selective pressure drive the emergence of drug resistance in cancer stem cells?

Finally, it will be critical to evaluate the clinical end points by which treatment success should be measured. The eradication of bulk disease is not likely to predict the efficacy of drug regimens for rare cancer cells. Therefore, the development of assays that measure the survival of cancer stem cells will be important for assessing the potential of new targeted regimens.

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Review Article

Methylation and colorectal cancer

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Abstract

Statistics rate colorectal adenocarcinoma as the most common cause of cancer death on exclusion of smoking-related neoplasia. However, the reported accumulation of genetic lesions over the adenoma to adenocarcinoma sequence cannot wholly account for the neoplastic phenotype. Recently, heritable, epigenetic changes in DNA methylation, in association with a repressive chromatin structure, have been identified as critical determinants of tumour progression. Indeed, the transcriptional silencing of both established and novel tumour suppressor genes has been attributed to the aberrant cytosine methylation of promoter-region CpG islands. This review aims to set these epigenetic changes within the context of the colorectal adenoma to adenocarcinoma sequence. The role of cytosine methylation in physiological and pathological gene silencing is discussed and the events behind aberrant cytosine methylation in ageing and cancer are appraised. Emphasis is placed on the interrelationships between epigenetic and genetic lesions and the manner in which they cooperate to define a CpG island methylator phenotype at an early stage in tumourigenesis. Finally, the applications of epigenetics to molecular pathology and patient diagnosis and treatment are reviewed. Copyright © 2001 John Wiley & Sons, Ltd.

Keywords: **colorectal cancer; DNA methylation; CpG island methylator phenotype**

Introduction

Human bowel epithelial tissue is particularly prone to cancer. At least 13% of the western population will develop a colorectal malignancy, which is now considered the leading cause of cancer death on exclusion of smoking-related neoplasia [1]. Moreover, the use of endoscopy has made the large intestine comparatively accessible and a step-wise breakdown in the faithful replication of the epithelial architecture is apparent macroscopically. Latter day research has accrued a vast amount of molecular data on the 'adenoma to carcinoma sequence', which has been used to evaluate the genetic basis of human cancer as a whole [2].

There are two established mechanisms for tumourigenesis. Loeb first proposed that tumour evolution is driven by genetic instability, with the generation of large numbers of random mutations and selection for clones exhibiting a mutator phenotype [3]. Soon after, Nowell's pioneering work on chromosomal aberrations in tumourigenesis led him to suggest that each cancer's individual genotype results from multiple rounds of clonal selection [3]. Both of these hypotheses are in keeping with Foulds' multi-step theory of tumourigenesis [4] and the hypothesized colorectal adenoma–carcinoma sequence. Vogelstein assembled strong support for a Darwinian model of adenoma–carcinoma progression [5] and further work on the genetic basis of human non-polyposis colorectal cancer (HNPCC) provided evidence for the theorized hypermutable phenotype [6].

While genetic alterations are a hallmark of human cancer, in many sporadic colorectal adenocarcinomas (CRA) the predicted accumulation of genetic events has been difficult to demonstrate [7]. Furthermore, there is little evidence for the incremental accretion of genetic events in ageing tissues that has been hypothesized to explain the exponential increase in the incidence of colorectal cancer with age [7]. Heritable 'epigenetic' changes in DNA methylation and/or chromatin structure were investigated some years ago to rationalize these inconsistencies [8]. However, in the rush to describe the genetic contributions to cancer, epigenetics was relegated to an almost non-existent role. More recently, this notion has been refuted and DNA methylation is now recognized as one of the most common molecular alterations in human neoplasms [9]. Specifically, the literature supports the concept that the loss of function of critical tumour suppressor genes is associated with age-related and cancer-specific hypermethylation in the promoter region [10].

A significant clustering of cancer-specific methylation events has been isolated in a subgroup of colorectal cancers [11]. If this small series is representative of the whole, then it seems probable that such a phenotype is not restricted to colorectal cancer [11]. Epigenetic analysis may influence future treatment regimens [9] and could even be used to map hitherto unknown tumour suppressor genes [12]. However, fundamental questions remain unanswered and many studies are under-powered, yet these concepts are already being implemented in the clinic.

In this review, the role of DNA methylation in cancer is examined and the evidence for a methylator

phenotype in colorectal cancer is reviewed. First, there is an overview of the mechanisms by which promoter methylation silences gene transcription, how methylation patterns are regulated, and how this is altered in ageing and cancer. The text stresses the interrelationships between epigenetic and genetic alterations, considers their position in tumourigenesis, and concludes with a discussion on the significance of epigenetics for the treatment of cancer and future research.

Colorectal cancer

In 1995, there were an estimated 32 140 new cases of large bowel cancer in the United Kingdom (UK) [13], representing *ca.* 9.4% of all incident cancer in men and *ca.*10.1% in women [14]. Eighty per cent of these cases present in those aged 60 years and older, with the rectum and the sigmoid colon accounting for two-thirds of the total [13]. Age-adjusted rates for men exceed those for women by up to 20% of the total in North America, although this difference is less marked in England and Wales [13]. Colorectal cancer is predominantly a disease of western countries, which average an incidence approximately 1.5-fold greater than elsewhere [14,15]. The mortality rates also rank colorectal cancer highly, with over 17 000 deaths each year in the UK, although there have been significant improvements in the 5-year survival rates since the early 1940s [13]. Positive risk factors for sporadic colorectal cancer include a western diet high in fat and red meat, obesity, and a high alcohol consumption [13,14]. Protective variables comprise a soluble dietary fibre load (from fruits and vegetables), a high level of physical activity, and the use of hormone replacement therapy and non-steroidal anti-inflammatory drugs (NSAIDs) [13,14]. These environmental considerations are complemented by genetic factors.

Approximately 5% of colorectal cancers [14] are due to recognized familial syndromes [16], for instance, HNPCC (the most common), familial adenomatous polyposis (FAP), Peutz–Jeghers' syndrome (PJS), familial juvenile polyposis [17], and others, which all predispose to malignancies at an early age. Different mutations in the adenomatous polyposis coli (*APC*) gene have distinct phenotypes, including Gardner syndrome (polyposis, osteomas, and epitheliomas), attenuated APC and congenital hypertrophy of the retinal pigment epithelium (CHRPE), and desmoid tumours in FAP patients. Turcot syndrome (colorectal polyposis with brain tumours) is also affected by the genotype of APC, but may have additional causes [18]. Moreover, an I1307K polymorphism, found in approximately 6% of Ashkenazi Jews, has been shown to elevate the risk of colorectal cancer [19]. Patients with ulcerative colitis (a form of inflammatory bowel disease) have a 2- to 8.2-fold increased risk of developing a colorectal malignancy, accounting for 1–2% of all cases [13,14]. Around 75% of colorectal cancer cases, though, arise sporadically in people aged 50 years and older who do not fall into a high-risk group [13], but individuals with a first-degree relative with the disease have around twice the average risk [13].

Vogelstein has mapped a series of genetic lesions that most colorectal cancers acquire as they progress to an invasive stage [5,20,21] (Figure 1), encompassing the five independent events that are required for a CRA to develop [22]. The epigenetic lesions considered below are discussed in the context of this model.

An epigenetic basis for colorectal tumourigenesis

CpG islands and DNA methylation

The CpG dinucleotide appears too short to be biologically significant [25], yet its properties are unique. Over 70% of the cytosine (C) bases that are covalently bound to guanine (G) (by a phosphodiester bond) are subject to 5′ methylation, with ensuing deamination to thymine (unmethylated cytosines deaminate to uracil) [25]. The defective repair of these anomalies, which are both carcinogenic lesions (as shown for the *p53* gene) [26], is responsible for the rarity of the sequence. Despite this, CpG dinucleotides are commonly enriched in discrete 'islands', 0.5–2 kb long, which are found in the 5′ region of 60% of genes (predominantly so-called 'housekeeping' genes) [25]. The accepted definition [27] describes regions of DNA greater than 197 bp, with a guanine/cytosine content above 0.5 and an observed or an expected presence of CpG above 0.6. The definition is one of sequence and not of function (i.e. it is not restricted to the promoter

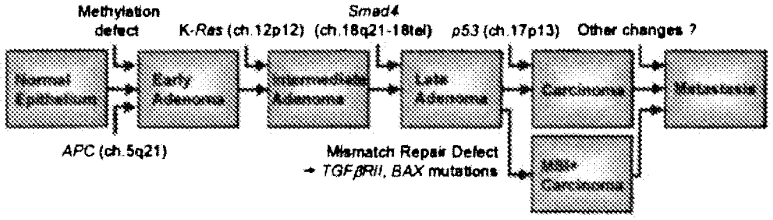

Figure 1. A genetic model for sporadic colorectal tumourigenesis. The linear accumulation of gene mutations and/or chromosomal anomalies is the best evidence for the Darwinian evolution of tumour progression [4,5]. It has since been extensively revised [19,23,24]. ch. = chromosome

CpG islands regulating gene expression), but is used for the purposes of this discussion. Most CpG islands (CGIs) are unmethylated, at least in young people, regardless of the expression of the associated gene, and are often flanked by methylated DNA [25]. These regions are in some way protected against methylation (despite being excellent substrates for DNA methyltransferase) and have been spared the evolutionary depletion of CpG dinucleotides seen in bulk DNA [11]. The reasons for this are unclear, but it is thought that the unmethylated state is a prerequisite for the involved genes to be maintained in an actively transcribed or transcription-ready state [28]. This state is perpetuated through mitosis [29] and, crucially for tumourigenesis, represents a heritable change.

Physiologically, DNA methylation within CGIs is essential for mammalian development [30] and serves an important function in X-chromosome inactivation [31] and genomic imprinting [32], as reviewed by Paulsen and Ferguson-Smith elsewhere in this issue. Methylation may also suppress transcriptional noise [33], defend the genome against retroviral elements, immobilize transposons [34], and control tissue-specific gene expression [35]. This links it to the maintenance of host defence and genomic integrity, but may compromise efforts at gene therapy [36].

The importance of CGI methylation as a method of tumour suppressor gene inactivation did not go unnoticed by molecular oncologists. However, this work is predated by studies of global hypomethylation at an early stage in colorectal neoplasia [8,37,38]. DNA methylation appears to suppress mitotic recombination and/or contributes to faithful chromosomal segregation during mitosis [39]. Chen *et al.* speculated that hypomethylation provides incipient cancer cells with a mutator phenotype, by destabilizing the karyotype and promoting loss of heterozygosity (LOH) [39]. Experiments on colorectal cell lines by Lengauer *et al.* [40] support this hypothesis. Hypomethylation might also favour a reduplication of oncogenes, given that an increase in the copy number of a transgene has been shown to accompany an increase in methylation and chromatin compaction and a reduction in gene expression [36]. Likewise, hypomethylation of proto-oncogenes may transform them into oncogenes, e.g. *Ras* [8], the multi-drug resistance 1 gene in acute myeloid leukaemia [41], and the neurotensin gene [42]. Similar data have been reported for c-*myc*, but the region examined was in the third exon and there was no attempt at a mechanistic link between methylation and gene expression [43].

In the late 1980s, hypermethylation of the CGI 5′ to the calcitonin gene (*CALCA*) was detected against a background of global hypomethylation in human colonic neoplasms [44]. While these changes appeared to be frequent, calcitonin is normally expressed only in neuroendocrine cells and is seemingly unimportant in tumourigenesis. Nonetheless, this gene was thought to offer a window on more consequential changes. Similar investigations in other human cancers led to the premise that there exist 'hot spots' for CGI methylation [45] and that this change could be responsible for the inactivation of tumour suppressor genes. The tight regulation of methylation during embryogenesis had previously been hypothesized to prevent the deleterious consequences of stochastic gene misfiring [46]. In a like manner, it appeared that the preservation of unmethylated promoters 5′ to tumour suppressor genes protected somatic cells against malignant change.

The discovery of allele-specific hypermethylation of the retinoblastoma tumour suppressor gene proved this theory [47]. The tumour for which Knudson first proposed his 'two hit hypothesis' (intragenic mutations and LOH) now displayed evidence for a new mode of inactivation [48], namely promoter hypermethylation, occurring at one or both alleles (Figure 2). This might explain the loss of expression of other tumour suppressor genes in cancers for which the accumulation

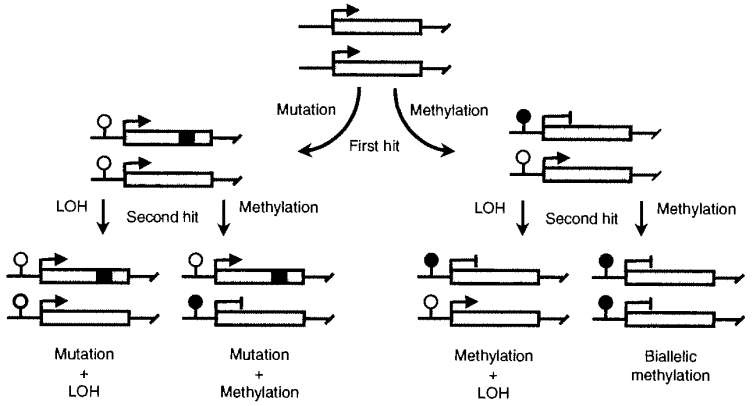

Figure 2. Knudson's 'two hit' hypothesis as revised by Jones and Laird [48]. In the original hypothesis, the two active alleles of a tumour suppressor gene are inactivated by mutations and loss of heterozygosity, respectively. Transcriptional repression, consequent upon DNA methylation of the promoter region, can occur at either stage and represents a third pathway to inactivation

of the requisite two genetic hits could not be demonstrated. Furthermore, in some instances methylation is the sole mechanism responsible for inactivation (e.g. $p16^{INK4a}$ in colorectal cancer) [49]. Many key tumour suppressor genes that are held responsible for hereditary cancer syndromes are also inactivated in sporadic cancers, in association with hypermethylation of a 5′ CGI. To illustrate, the human *MutL* homologue (*hMLH1*), which is mutated in the germline of certain HNPCC kindreds, is frequently methylated in sporadic colorectal cancer [50]. Certain carcinogens appear to affect the pattern of DNA methylation [51–53], bolstering what is now a well-established transformation. This inactivation accompanies the hyperproliferative state observed in neoplasia and ageing [54] and by extension, hypermethylation could represent a pro-tumourigenic lesion.

Methods

Much effort has been directed towards adapting standard biochemical and genetic protocols to the study of epigenetics. The procedures used are diverse, complex, and often flawed to greater or lesser degrees. Moreover, they contribute to, if not account for, many of the controversies in the literature. It is therefore essential to review briefly the most widely used techniques.

The method chosen depends on the application. Several approaches have been used to scan the genome for methylation changes and to identify putative tumour suppressor genes. Different methodologies are employed to detect methylation at CpG sites in specific target areas of these genes (Table 1). Each approach may use one or other of two principal techniques. First methylation-sensitive restriction enzymes can distinguish methylated and unmethylated sequences by their ability to selectively cleave DNA at one or more methylated CpG sites. The products of DNA digestion are thus methylation-dependent [55]. Lately, this has been superseded by bisulphite modification [56]. Sodium bisulphite is a mutagen that selectively deaminates unmethylated cytosine residues to uracil, leaving the 5-methylcytosine bases intact [56]. Both techniques are then followed by assays for the specific markers of methylation, i.e. the products of DNA digestion or the remaining cytosine bases [55].

Problems and limitations have been reported with all protocols. Some require a relatively large quantity of good quality DNA, excluding the study of paraffin-embedded samples and small lesions [57]. Restriction analysis is plagued by false positives because of incomplete digestion by the methylation-sensitive enzymes [58]. Further, only a limited number of CpG sites within an island can be analysed [58]. It is preferable, therefore, to restrict with enzymes that have at least two CpG sites in their recognition sequence (e.g. *Not*I, *Sac*II, *Eag*I, *Bss*HII, etc.) to increase sensitivity [55]. Many of the polymerase chain reaction (PCR)-based techniques are fraught with quantitation problems, as well as PCR bias, since differentially methylated DNA samples sometimes amplify with greatly differing efficiency [55].

Tumour heterogeneity and contamination of tumour samples with normal cells will distort qualitative results (see below) [55]. Microdissection, adequate sample numbers, quantitative methods, and a critical eye are thus essential. In addition, continuous selection of cell lines in culture favours their 'artifactual' methylation [65]. To illustrate, the 5′ CGI of the *14-3-3* σ gene is methylated in the colorectal cell line COLO320DM, but not in CRA tissue samples [66]. Therefore caution is advised when interpreting methylation patterns in these models.

Age-related methylation

Age-related methylation (Table 2) is a progressive process during which methylation in neighbouring *alu* repeats encroaches on certain promoter-region CGIs over the lifetime of 'normal' tissue, favouring neoplasia by continuous selection [67].

Age is the principal function of colorectal adenocarcinoma incidence [17] and cancer is a disease of the genes. However, genetic lesions have not been documented in the context of ageing tissues [7]. Consequently, theories concerning telomere dysfunction, a deterioration in immunity, epigenetic dysregulation, and altered stromal

Table 1. Methods used to detect methylation

Global detection	
Restriction landmark genome scanning (RLGS)	RLGS is the most reliable method for estimating the genomic frequency of CGI methylation [59].
Representational difference analysis [55]	
Methylation-sensitive arbitrarily primed PCR [55]	MCA is the most recent addition, developed as a high-throughput method and as a first step for cloning differentially methylated CGIs [57].
Methylated CpG island amplification (MCA)	
Individual CpG island analysis	
Post-restriction Southern blot analysis [49]	The gold standard for the quantitative analysis of methylated CGIs.
Restriction PCR [60]	PCR amplification from primers flanking the restriction sites.
Bisulphite sequencing [61]	Bisulphite modification is also employed in the methods cited below.
Methylation sensitive PCR (MSP) [58]	Rapid, reliable and sensitive (to 0.1% of methylated alleles).
Real-time MSP [62]	Yields quantitative results.
Bisulphite PCR restriction analysis (COBRA) [63]	The additional restriction step yields more quantitative results.
MethyLight (fluorescence-based real-time PCR) [64]	Highly sensitive and quantitative to mono/bi-allelic methylation.
Methylation-sensitive single nucleotide primer extension [55]	A dot-blot analysis makes it relatively fast and quantitative.

Table 2. A summary of published data on methylation in the colonic epithelium [see text and ref. 55]

Gene	Methylation in primary CRAs (%)	Map	Comments	Function
Promoters methylated in sporadic cancer only				
APC	18	5q21	Methylation favours wild-type alleles	Principal gatekeeper
CACNA1G	35	17q22	A MCA clone	Apoptosis and proliferation?
CALCA	31–93	11p15	One of the first demonstrated in cancer	Bystander gene
COX-2	15	1q25.2–3	Associated with wild-type (wt) *p53*	Tumor growth regulation
HIC-1	80–100	17p13.3	Cloned using RLGS; associated with wt *p53*	Transcription factor
hMLH1	75 MSI+	2p22	A consequence, not a cause of CGI methylation	Mismatch repair
LKB1	8	19p13.3	Methylated in 18% of PJS hamartomas	Ser/Thr kinase
MGMT	26–38	10q26	Assoicated with G→A transitions in K-*Ras*	Direct repair
$p14^{ARF}$	28–32	9p21	Not associated with $p16^{INK4a}$, *p53*, *Ras* or *MGMT*	p53 regulator
$p16^{INK4a}$	28–55	9p21	Associated with wt *p53* and K-*Ras* mutations	Cell-cycle and senescence
THBS1	24	15q15	Regulated by p53 and Rb	Angiogenesis
TIMP3	28	22q12.1–13.2		Metastasis
WT1	68–74	11p13	Methylation not correlated with expression	Transcription factor
Promoters methylated in ageing large bowel and sporadic colorectal cancer				
ER	100	6q25.1	Epigenetic profile is altered by carcinogens	Growth and differentiation
CSPG2	70	5q12–14		Regulated by wt *Rb*
IGF2	100	11p15.5	Imprinted gene	Growth factor
MYOD1	100	11p15.4		Bystander gene
N33	91	8p22	Tissue-specific methylation	Bystander gene
PAX6	70	11p13		Bystander gene
RARβ2	43	3p24		Growth factor signalling
Promoters unaffected in ageing large bowel and sporadic colorectal cancer				
14-3-3σ		1p	Methylated in a colorectal cell line only	G_2/M regulator
Fas		10q		Apoptosis
GSTP1		11q13		Detoxifying enzyme
Other *MMR* genes			e.g. *hMSH2*	Mismatch repair
$p15^{INK4b}$		9p21		Transforming growth factor (TGF) β signalling
SMAD4		18q21		TGFβ signalling
Hypomethylation in sporadic colon cancer				
Global	100		Associated with genetic instability	Chromosomal segregation during mitosis

milieu have all been cited to explain the exponential increase in tumour incidence [7]. Genomic hypomethylation was the first epigenetic observation in ageing tissues and was originally proposed to function as a 'counting mechanism' for cellular senescence [29].

Issa *et al.* [68] first discovered methylation of the oestrogen receptor gene (*ER*) in normal colonic epithelium. The lesion is now considered to modulate growth and differentiation in normal colonic enterocytes (i.e. *ER* is a candidate tumour suppressor gene) [68]. All colonic tumours examined, including small adenomas, had extensive *ER* methylation [68]. Accordingly, the incremental methylation observed in 'normal' tissues was highest (75%) on the left side of the colon, where tumour incidence is greatest [13]. It was hypothesized that ageing normal colonic mucosa acquires a sub-population of cells with hypermethylation of the *ER* promoter CGI. Subsequently, linear increases in the density of this methylation, as a function of maturity (0.2–0.3% per annum), might portend progression towards malignancy [68]. Successive investigations have backed this finding in several other promoter-region CGIs, for instance, those associated with the *IGF2* (insulin-like growth factor 2), *MYOD1*, *N33*, *PAX6*, and *CSPG2* (Versican) genes [10,69,70]. Furthermore, the studies allege that the age-related loci are highly tissue-specific [62].

Mature stem cells could conceivably have a markedly different physiology than their younger counterparts [67]. To cite an instance, preliminary findings alluded to by Issa [71] show that the colonic epithelium ages prematurely in patients with inflammatory bowel disease. There are three-fold higher levels of age-related methylation in apparently normal mucosa and five-fold higher levels in dysplastic mucosa, compared with controls [71]. These controls should be from an appropriate, purified tissue, since stromal cells may be less susceptible to age-related methylation than epithelial tissue [70]. Unheeded, this could lead to an underestimation of the relative contribution of age-related methylation to the total incidence of hypermethylation in neoplasia [72].

In contrast, some genes, e.g. *THBS1* (thrombospondin-1), *HIC-1* (hypermethylated in cancer 1), and *CALCA*, show no evidence of methylation in any normal colonic mucosa, but are *de novo* methylated in colon cancers (see below) [70,73]. Therefore, age-related methylation is gene-specific and could account

for up to 73% of the hypermethylated loci in CRAs [10]. This manifests as an exponential increase in the incidence of cancer as the cells age [67].

The CpG island methylator phenotype

The concept that there exist two distinct patterns of CGI hypermethylation, associated with ageing and cancer respectively, has been confirmed recently. It was initially unclear whether this process was wholly stochastic with ensuing selection pressure over time, driven by the inactivation of anti-oncogenes, or whether a clustering of epigenetic events defined a subset of colorectal tumours. The methylation of promoters has been correlated with wild-type genes (e.g. $p16^{INK4a}$) and progressive methylation in neoplastic cells may help to overcome growth arrest [74]. In due course, the protection afforded CGIs is sporadically transcended, driving clonal expansion by a significant growth advantage [75]. However, this theory cannot account for the many 'bystander' genes that are methylated in cancer but do not provide a selective advantage (e.g. *CALCA*) [44].

The development of methylated CpG island amplification (MCA) provided researchers with the opportunity to assess whether there was any evidence to back the 'clustering' hypothesis [10]. Toyota *et al.* analysed 26 new loci, termed MINT (methylated in tumours), all of which have CGIs as defined previously [10]. The newly cloned loci fell into the two groups, age-related and cancer-specific. Of these, the majority were methylated in 'normal' ageing epithelial cells, as well as in colorectal lesions (type A methylation), whilst only seven were methylated exclusively in colorectal tumours (type C methylation) [10]. Dissection of the results enabled the authors to form a nascent definition for the CpG island methylator phenotype (CIMP) [10]. Of the 50 CRAs and adenomas, a group with a high level of type C methylation was catalogued as CIMP+, wherein all the tumours had methylation of three or more loci simultaneously (a mean 5.1 loci per tumour) [10]. In the remainder, the methylation of type C clones was extremely rare (a mean 0.3 loci per tumour) [10]. Type A methylation was not significantly different between the two groups [10]. There was also excellent concordance between the epigenetic silencing of three known tumour suppressor genes ($p16^{INK4a}$, *hMLH1*, and *THBS1*) and CIMP+ status. Subsequently, work on cyclooxygenase (COX)-2 (see below) correlated its CGI methylation to the CIMP ($p<0.01$), even though exclusivity was not demonstrated [10]. Contrary to prior work [40,77], the CIMP+ and microsatellite instability phenotypes (MSI+) did not exclusively co-segregate (see below) [10].

Despite the limited series, significance was reached for relationships between the CIMP and tumours located in the proximal colon ($p=0.003$), although numbers probably precluded judgement on any associations with age and/or gender [10]. In contrast, type A methylation is found predominantly in tumours of the distal colon [68] and yet it still prevails in proximal tumours [10]. However, in spite of the high prevalence of type A methylation events in colorectal tumours (seen in 80–100% of cases here), type C events are more critical, as they involve considerably more important tumour suppressor genes [10].

The large number of loci examined, the breadth of techniques employed to confirm the findings, the lack of a significant difference in type A methylation between CIMP+ and CIMP– tumours [10], and the backing of previous work [40] all support the existence of such a phenotype. Molecular investigations on an epidemiological scale, though, will be needed to determine the proportion of sporadic cancers that are CIMP+. A follow-up study defined distinct genetic profiles in colorectal tumours with or without the CIMP, which may provide a blueprint for the phenotype's expression profile [48,78]. The transcriptome of the CIMP is not restricted to methylated genes. Indeed, DNA methylation may inactivate several caretaker genes, making it a hypermutable phenotype of sufficient order to meet Loeb's hypothesis [3]. The term 'epi-mutator phenotype' is, therefore, more accurate than 'CpG island methylator phenotype'.

The molecular biology of DNA methylation

'Belts, braces, and chromatin' [35]

Hypermethylation of CGIs in cancer is strongly associated with transcriptional silencing of the genes concerned [9]. The causation or association nature of this relationship is evaluated below, using Hill's predictors of causality [79]. A non-linear relationship between the lack of repression observed at low densities of methyl CpG and repression at higher densities [80] provides a biological gradient and the literature is consistent, plausible, and coherent. Even so, 'association' is the operative term here and no one study has determined whether DNA methylation is a vital player or an epiphenomenon with a weak modulatory role at best. Several lines of evidence, though, indicate that the relationship between methylation and gene expression is not just correlative.

Could DNA methylation prevent the binding of basal transcription machinery or ubiquitous transcription factors, which require contact with cytosine in the major groove of the double helix? This is a strong possibility, given that most mammalian transcription factors have GC-rich binding sites and many have CpGs in their DNA recognition elements [35]. In addition, binding by several of these factors is retarded or even abolished by the methylation of CGIs (Figure 3a), possibly due to the protrusion of methyl groups into the major groove of the DNA helix [35], or by a putative influence on nucleosome positioning and mobility [81,82]. The reduction in affinity of certain factors, however, is often insufficient to account for the inactivity of promoters *in vivo* [83]. To date, the

evidence consigns this theory to a minor role, as several densely methylated genes can be transcribed even when chromatin and/or methyl-CpG binding proteins are absent [35].

An alternative means implicates observed changes in the architecture of the nucleosomal core that are linked with CpG methylation (reviewed in ref. 84). Histone hyperacetylation is an essential feature of transcriptionally competent euchromatin. Two molecules of each of the four core histones (H2A, H2B, H3, and H4), together with a 146 bp DNA sequence, make up a nucleosome core (the structural element of chromatin). Acetylation greatly reduces the affinity of the protein hub for its encircling double helix and disrupts the orderly packing of nucleosomes in arrays [84]. Experimental data confirm that these breaches allow access to *trans*-acting factors without compromising the architecture of the unit [84]. Higher-order chromatin

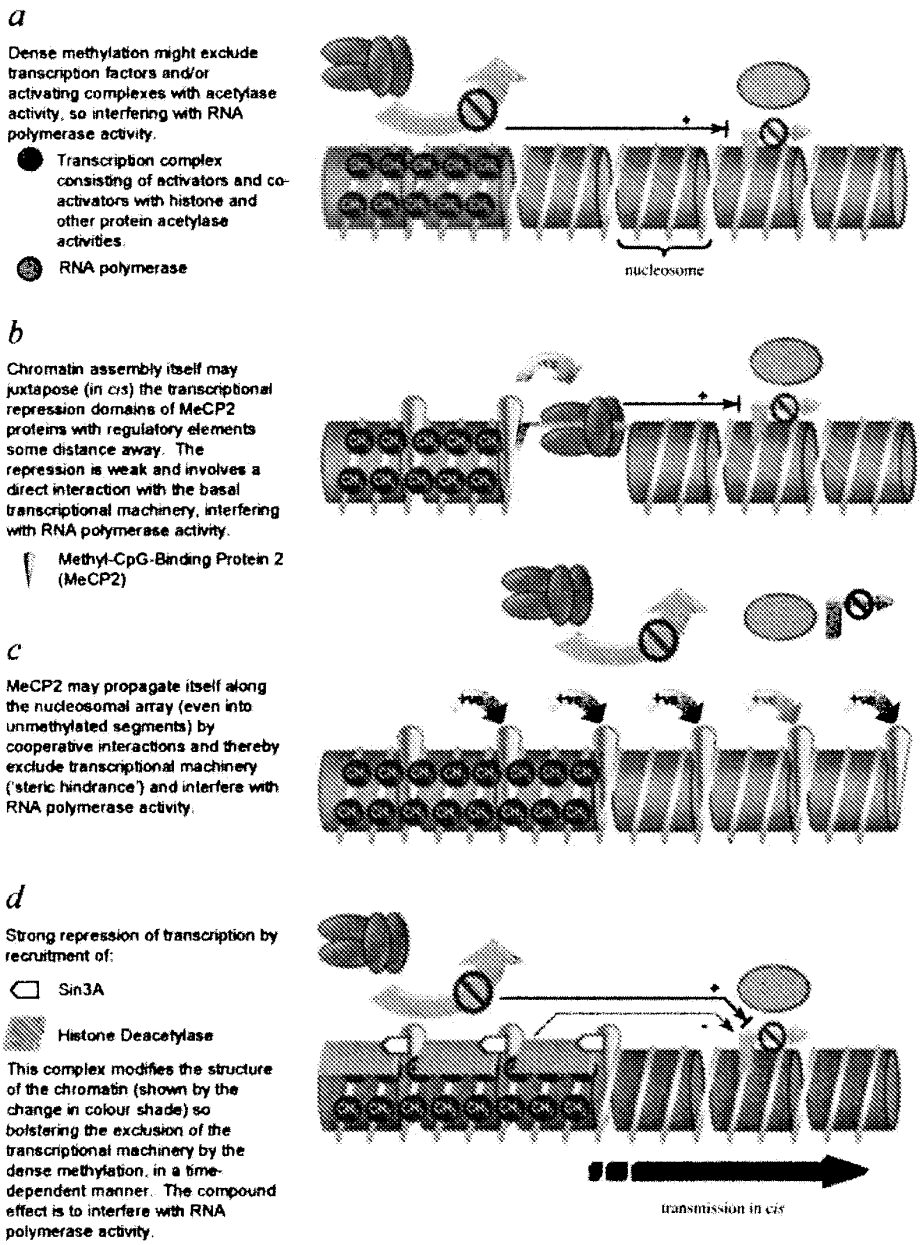

Figure 3. Speculative models (a–d) for the molecular mechanisms by which DNA methylation may result in transcriptional silencing [35,84,90]:

structures also hinge upon the folding of linker DNA, directed by elements such as the linker histones, e.g. H1 [84]. Consistently experiments have found that unmethylated chromatin is enriched in hyperacetylated core histones and deficient in linker histones [84]. In contrast, heterochromatin assembled on artificially methylated DNA associates with hypoacetylated core histones, is refractory to nuclease or restriction endonuclease digestion, and remains transcriptionally silent [84]. Thus, hypomethylation of the promoter is associated with transcriptionally competent euchromatin and may be involved in the maintenance of transcription through the gene.

A lucid account of the observed changes awaits the identification and characterization of the multifaceted methylation machinery. Three complexes are known to bind to methylated CpG dinucleotides [25]. The most well defined are methyl-CpG-binding protein 1 (MeCP1) and MeCP2 [85,86]. MeCP2 is a polypeptide with both a methyl-CpG-binding domain (MBD) and a transcriptional repression domain (TRD) [79]. It is capable of binding to both naked methylated DNA and to the same helix when assembled into chromatin [79]. The TRD can interact directly with the basal transcription machinery to silence transcription over a distance of several 100 base pairs (aided by the compact structure of chromatin) (Figure 3b) [79]. MeCP2 may also append more effectively to nucleosomal rather than to naked DNA, propagating itself into unmethylated segments by cooperative interactions and thereby excluding the transcriptional machinery ('steric hindrance') (Figure 3c) [84,87]. Concomitantly, by 'interference', the presence of MeCP2 blocks the loading or activity of RNA polymerase [87]. On the next level, the TRD enlists Sin3A, which couples it to a histone deacetylase complex (Figure 3d) containing the enzymes histone deacetylase (HDAC) 1 and/or HDAC2 [88,89]. The stability of transcriptional silencing, achieved by repressive nucleoprotein complexes is transmissible in *cis* (once a threshold of methylation density has been reached), precluding a strict necessity for DNA methylation within the promoter [90] or at transcription factor binding sites [91]. This mechanistic bridge between the two global systems of gene regulation (DNA methylation and histone acetylation) and the dependence of transcriptional silencing on chromatin assembly is plausible but simplistic; a definitive mechanism awaits clarification of a number of points. Sin3A retains some ability to repress transcription in the absence of histone deacetylases, MeCP2 may favour the local deacetylation of certain transcription proteins [92], and other proteins co-fractionate with MeCP2 and Sin3A, but their contribution is unknown [89]. Moreover, cells deficient in MeCP2 (e.g. HeLa cells) are able to repress methylated *trans*-genes [93].

On searching EST (expressed sequence tag) libraries for the MBD of MeCP2, a family of methyl binding proteins (MBD 1, 2, 2b, 3, and 4) was isolated [86]. MBD1 is a lone protein, selectively binding to methylated DNA and repressing transcription from a naked methylated promoter *in vitro* [35]. MBD2 is a component of MeCP1, together with histone deacetylases and various associated proteins [93], and MBD3 resides in a distinct complex, forming part of the Mi-2/NuRD deacetylase [94]. Mi-2 is a member of the SWI2/SNF2 superfamily of ATPases that disrupt histone –DNA interactions [35] and, in this context, it enhances deacetylase activity through an associated protein, RbAp48 [35]. However, the relevance of Mi-2/NuRD to the repression of transcription in mammalian systems is questionable [86,94].

Despite the seeming abundance of repressive proteins, they are vastly outnumbered by methyl-CpGs (*ca.* 2×10^7 in the genome) [35]. Therefore, in all probability, these distinct complexes have equally distinct functions. MeCP1 may be involved in transient repression [93]; Mi-2 is directed to regions of the genome discrete from MeCP2 [94]; and the latter is believed to induce stable, long-term repression [93]. Finally, MBD4 is thought to regulate methylation patterns by directing the repair of deaminated 5-methylcytosine residues (which generate guanine·thymine mispairs) through its interactions with hMLH1 [95].

In summary, the data support a progression of events, beginning with methylated DNA sequestering methyl-CpG-binding proteins. Thereafter, the local deacetylation of histones condenses the chromatin, providing a molecular lock to turn transcription 'off' [46]. This logic goes some way towards establishing causality between DNA methylation, the binding apparatus, and transcriptional repression.

How do these findings relate to genes that are hypermethylated in cancer?

Drug treatments have been used extensively to investigate epigenetics. 5-Aza-2′-deoxycytidine (deAZA, a demethylating agent) and trichostatin A (TSA, an inhibitor of histone deacetylase) were employed by Cameron *et al.* [96] to investigate the relative roles of methylation and histone deacetylase activity. Their work on cell lines (for instance, the colorectal cell line RKO) established that dense CGI methylation is dominant for the stable inhibition of transcription from endogenous promoters [96]. Thus, the apparent synergy between the two events may be extended to include oncogenesis, but the relationship does not hold in all instances [96]. A dynamic model of promoter regulation helps to clarify this. Cellular signals harmonize the competing influences of activating and repressing protein complexes to control the level of expression from most transcribed or transcription-ready genes [97]. Gene expression, therefore, results from the relative industry of acetylases (e.g. p300, pCAF, and CBP) and deacetylases [98], and DNA methylation might favour repression by two collaborative events [84]. The dense methylation may exclude activating complexes with acetylase activities and the methyl-CpG-binding proteins might tether complexes

that contain transcriptional co-repressors and histone deacetylases. Indeed, TSA could only induce gene expression when some degree of demethylation had been achieved, which in turn allowed the return of transcriptional complexes with acetylase activities [96,99]. To substantiate this, further work is needed to investigate how the constituents of the transcriptional complexes compare for unmethylated versus oncogenically methylated endogenous promoters [99]. A feasible explanation must also contend with gene promoters (e.g. *MGMT*) in which only a limited amount of heterogeneous methylation is sufficient to silence expression [100].

A family of DNA methyltransferases

During mammalian embryogenesis, methylation needs to be established, maintained, and removed in a dynamic process, which has to be tightly regulated if the cell is to avoid the deleterious consequences of hypo- or hyper-methylation [101]. To date, three DNA methyltransferases (DNMT1, 3a, and 3b) have been cloned from mammalian cells [87,101,102]. Each catalyses the transfer of methyl groups from *S*-adenosyl-methionine onto the 5′ position of a cytosine base residing in a CpG dinucleotide [75]. The best-studied process is maintenance methylation, which ensures that DNA methylation patterns continue in perpetuity on the genomes of daughter cells [101]. DNMT1 has been assigned the leading role in this operation, as it has a 5- to 30-fold preference for hemimethylated substrates [101]. The enzyme is sequestered by the replication machinery (specifically by proliferating cell nuclear antigen, PCNA) [75], possibly with MeCP1 or MeCP2, to re-establish symmetrical CpG methylation and an identical chromatin configuration on the daughter genomes [35]. DNMT3a and 3b show only weak maintenance activity and may be the *de novo* enzymes necessary for normal embryonic development [103]. However, the fidelity of DNMT1 to maintenance work is low and the activity of DNMT1 on unmethylated DNA substrates outweighs the other DNMTs ascribed to *de novo* methylation [101]. Indeed, overexpression of DNMT1 drives the *de novo* methylation of susceptible CGIs, implying that DNMT1 can contribute to tumour progression [104]. A putative human demethylase has also been identified recently as the MBD2b gene product [105], demonstrating that this is a dynamic process, which may be deranged as much from reduced active demethylation as increased active methylation [106].

With reference to cancer, DNMT1 has been linked with cellular transformation, several oncogenes, and tumour suppressor genes [75]. The activity of DNMT1 (as well as DNMT3a and DNMT3b) is elevated in colorectal cancer cells, increasing in a linear fashion with tumour progression [107–109]. On reconciliation with the increase in cell proliferation, though, these changes are merely compatible with other markers of cell proliferation [110]. However, the induction of DNMT1 induces transformation of NIH 3T3 cells [111] and deAZA [112], biallelic DNMT1 knockouts [39], and antisense cDNA directed against DNMT1 mRNA [113,114] all discourage tumourigenesis. Transcription of *DNMT1* is regulated by Rb and the Ras–AP-1 signalling pathway (involving Fos/Jun) [115–117]. Hence, oncogenic signalling may be responsible for the raised expression. Nonetheless, the putative transcription start sites could only yield a truncated protein, which is not observed in somatic cells [101]. Initially, researchers tried to prove the most obvious association that the increase in DNMT1 expression was responsible for the aberrant methylation, but this seemed not to be the case [118,119]. Other DNMTs are probably responsible for this occurrence, but what of DNMT1's oncogenicity?

In a recent review [75], Szyf *et al.* proffered that DNMT1 transforms cells by a mechanism independent of its main biochemical function. They supposed that the requirement of DNMT1 for the assembly of a replication-competent replication fork has evolved to assign DNMT1 a role in cell proliferation [75]. DNMT1 competes with $p21^{WAF1}$ for PCNA [75,120–122], such that when $p21^{WAF1}$ is up-regulated, in response to growth arrest signals, it cannot form a binary complex with PCNA, which is sequestered by the high levels of DNMT1 in cancer cells [75,107]. The DNMT1-mediated sequestration of PCNA affects cyclin-dependent kinase (CDK) and cyclin levels, which in turn influence the phosphorylation status of the Rb protein and participate in a positive feedback loop on $p21^{WAF1}$ expression [75]. Ultimately, this allows cell growth to continue in spite of the accumulating DNA damage, and thereby it engenders a mutator phenotype.

Identifying the oncogenic flaw

The predicted mutations in the DNA methylation machinery have not been found in neoplasia, but mutations in DNMT3b and MeCP2 are held responsible for ICF syndrome (immunodeficiency, centromeric instability, and facial anomalies) [103,123,124] and Rett's syndrome [125], respectively. However, age-related methylation is so universal that it is unlikely to be caused by a similar mutation and is probably an upshot of a physiological process [55]. There are several possibilities: first, specific carcinogen exposures (plutonium and nickel) have been linked with DNA methylation [51–53]. Quite how the carcinogens exert their effect is unknown, but some believe that they 'seed' chromatin condensation, which is later stabilized by DNA methylation [53]. Thus, it is possible that the cumulative exposure to such agents is responsible for the changing epigenetic profile of 'ageing' genes and even cancer-specific methylation loci [51]. Second, there may be communication between DNA methylation and other genetic changes which accompany ageing [67], e.g. telomere shortening or telomerase activity [7]. Third, ageing is associated with changes in

gene expression, some of which may affect methylation. Indeed, abated gene expression may set up a gene promoter for hypermethylation [67]. This was proposed for the *ER* as a function of reduced circulating oestrogen levels with age [68].

Changes in the biochemistry that sets the patterns of mammalian methylation appear most consequential. Methylation centres, consisting of highly repetitive sequences, e.g. *alu* repeats, exist throughout the genome and serve as a focus from which *de novo* methylation proceeds both upstream and downstream in *cis* [126]. The CGIs of certain genes, such as *MYOD1* [70], are thought to lie very close to these centres (found in *MYOD1*'s first intron and second exon). From here, the methylation overrides the inadequately protected promoter as the tissue ages, with a resultant loss of expression (Figure 4) [67].

Age-related methylation is widespread, but by contrast, the genes *de novo*-methylated in human cancer show significant clustering in the colon [10], suggesting that the CIMP is secondary to an acquired defect in the CGI methylation/demethylation process [55]. There are several theories concerning this and the truth probably lies in a coalition of hypotheses [55]. The literature favours the protection of CGIs by 5′ and 3′ *cis*-acting elements, e.g. Sp1 binding sites, which form methylation boundaries between the surrounding *alu* repeats and the gene promoter. Sp1 elements are thought to attract *trans*-acting factors (other than Sp1 itself), which regulate transcription, exclude *de novo* methylases, or possibly direct the DNA demethylase to promoter CGIs for the correction of aberrant *de novo* methylation [75,127]. Methylation spreading occurs in the absence of DNMT1, indicating that the enzyme responsible is probably one of the *de novo* methylases (DNMT3a or DNMT3b) or an undiscovered DNMT [126]. Nevertheless, the properties of DNMT1 contradict this. DNMT1 preferentially *de novo*-methylates regions of lower methyl-CpG density at the borders of the methylation centres rather than the denser core [128]. Its overexpression can also erode the protective influence of flanking Sp1 sites, although DNMT1 activity is similar between the CIMP+ and CIMP– tumours (Figure 4) [10]. This is reconciled by the theory that the CIMP may be caused by a decrease in *trans*-acting factor activity, as alleged for certain breast cancer cell lines [129]. Moreover, buffering insulator elements can protect against the hypermethylation, hypoacetylation, and extinction of expression of *trans*-genes [130].

The co-existence of global hypomethylation and focal hypermethylation in cancer is paradoxical. However, emerging studies on the molecular structure of DNMT1 and its manifold roles in the cell may soon resolve this. DNMT may be directed to specific sequences by artificial fusion with a zinc-finger protein [131]; this might reflect a similar physiological mechanism. DNMT1 co-purifies with Rb, the E2F1 transcription factor, and HDAC1 [132]. This complex is capable of repressing transcription from promoters containing E2F1-binding sites, with implications for the targeting of methylation, transcriptional control, DNA replication, and tumourigenesis [132]. Any defects in these proteins may disturb the regulation of this complex, causing DNMT1 to mis-localize and accounting for the synchronous global hypomethylation and focal hypermethylation [132]. In a negative feedback loop, the methylation of an AP-1-dependent regulatory element in *DNMT1* acts as a sensor of the DNA methylation capacity of the cell [133]. This regulation might be aberrant in neoplasia [133]. Should the DNA demethylase exhibit different substrate specificities to DNMT1 and if its activity is dysregulated in cancer, the combined effect may account for this paradox [134]. Other proposals implicate the aforementioned components of histone acetylase/deacetylase dynamism [127].

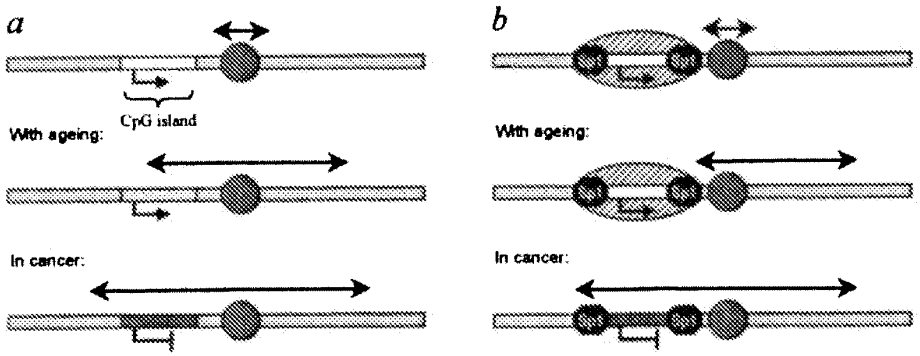

Figure 4. A model of the two forms of methylation in cancer. (a) Type A methylation. The spreading of methylation from a methylation centre (circle), composed of *alu* repeats, gradually proceeds in *cis* (both 3′ and 5′) with ageing. Eventually it overrides the weakly protected promoter CpG island, silencing transcription and favouring hyperproliferation. (b) Type C methylation. Certain genes are protected from ageing by *trans*-acting factors (oval), which bind to Sp1 sites at the borders of the CpG island. However, a defect in the methylation machinery, acquired during tumourigenesis, withdraws this protection or renders it ineffective, allowing methylation to spread through the promoter and silence the gene [8,10,65].

Of distinction is a member of the SWI/SNF family (chromatin-modelling engines), which facilitate gene expression [35] and are highly conserved from yeasts to plants to mammals [135]. When mutated in *Arabidopsis*, the changes in DNA methylation approximate those typical of human cancer [136]. Could the human homologue be culpable in neoplasia?

Seemingly, a lesion in the system that regulates maintenance and/or *de novo* methylation is the oncogenic flaw responsible for the epigenetic changes noted in cancer. While this lesion has not been identified, other members of the methylation machinery show curious parallels with cancer. The Mi-2 complex contains a p80 polypeptide similar to the metastasis-associated gene (*MTA1*), the overexpression of which is correlated with invasion and metastasis in gastrointestinal carcinomas [94,137,138]. Furthermore, MBD2 is identical to NY-CO-41, a human cancer antigen, which is the target of autoantibodies from colon cancer patients [94].

Evidently, additional studies are required to clarify the intricate and complicated basis of epigenetics, both in terms of its normal physiology and how this is altered in ageing and cancer.

Epidemiology and epigenetics

Epidemiological data implicate hormonal and reproductive influences in the aetiology of CRAs in women [15]. In particular, post-menopausal hormone replacement therapy reduces the risk for CRAs and adenomatous polyps [15]. The hormones may operate directly on the tumour to control growth, as supported by age-related methylation of the *ER* promoter [107], or they may affect the metabolism of bile acids, which have promoting effects on the colonic epithelium [139]. Women have an excess of right-sided CRAs and develop their cancers at an earlier age than men [15]. Notably, though, minority groups in the US have a pronounced increase in right-sided CRAs among older women [140]. Taken together with the clinicopathological data described above, the CIMP may represent distinct anatomical and gender-related subgroups, for whom clinical trials could potentially optimize a treatment regimen (see below).

Given the well-documented associations between dietary factors and the risk of colorectal cancer, a relationship between diet and epithelial DNA methylation seems likely. Selenium, an essential trace element in humans, has received attention for its possible role as an anti-carcinogen [141]. Work done on cellular models prompted the authors to suggest that a dietary deficiency in selenium promotes colon tumourigenesis by way of an effect on DNA methylation [141]. Selenium is a donor for *S*-adenosyl methionine and hence it depletes levels in the cell by enzyme-mediated biomethylation [141]. Therefore, selenium may prove to be an important, exogenous regulator of both maintenance and *de novo* methylation. However, in view of its associations with colorectal cancer, the methyl-donor pathway is the most promising candidate. It involves MTHFR (5,10-methylenetetrahydrofolate reductase) and is affected by dietary sources of folate, vitamins B_6 and B_{12}, and methionine [15]. This cyclical reaction catalyses the remethylation of homocysteine to methionine [142], a precursor of the DNMT methyl donor *S*-adenosyl methionine [101]. Preparatory work on animal models paved the way for a large-scale epidemiological study [143], but it offered 'only limited support for previously reported associations between dietary factors involved in DNA methylation and risk of colon cancer'. Further work, though, found an association between a deficient intake of folate, vitamin B_6 and vitamin B_{12}, a homozygous C677T polymorphism in the MTHFR gene, and a doubling in the risk of colon cancer [142]. The article also demonstrated a site and age specificity implicative of the CIMP, but these interactions have not been confirmed by large-scale epidemiological studies.

Tumour suppressors that are methylated in a cancer-specific manner

In contrast to age-related methylation, the loci involved in cancer-specific CGI hypermethylation are inactivated exclusively in neoplastic tissues (unless otherwise stated, the CGIs mentioned are unmethylated in normal colorectal epithelium). However, cancer specificity alone does not prove that the aberrant methylation of a gene promoter is functionally relevant. In assessing the papers discussed below, two fundamental questions have been asked [144]: (1) Is methylation at this locus coupled to transcriptional silencing from the promoter? (2) Does it matter for tumourigenesis? Some genes are bystanders (for instance, *CALCA*) [44] and do not contribute to the transformed phenotype themselves, but they can be misleading in this respect. To ascertain the functional significance of the patterns of hypermethylation in tumour suppressor genes, the researchers must demonstrate a selective advantage [145], which is often lacking from published material. In lieu of a clear indication of biological importance, this review adheres to the criteria laid out by Herman [144] in its appraisal of aberrant methylation.

The principal gatekeeper

One of the earliest steps in the development of CRAs is inactivation of the adenomatous polyposis coli gene (*APC*), the principal gatekeeper in intestinal epithelium [6]. A classical tumour suppressor gene, it was identified from the co-segregation of mutant alleles in kindreds affected with FAP and is responsible for an increased rate of tumour initiation in these families [6]. Macroscopically, this is evident as hundreds to thousands of polypoid colorectal adenomas in affected individuals [2,6,18]. In sporadic tumourigenesis, *APC* mutations present as foci of dysplastic aberrant colonic crypts and are found in 80% of adenomas [146]. Its

supervision of the equipoise between birth and death in the colon is chiefly mediated by the sequestration, targeted phosphorylation, and degradation of β-catenin [6,146,147], a member of the WNT signalling pathway [148]. The accumulated β-catenin both interacts with cadherins and forms heterodimeric transcription factors with members of the Tcf/Lef family, leading to growth promotion [147,148] by the activation of c-*myc*, etc. [24]. Inactivation of *APC* may also cause neoplasia by its effects on cell death, since expression of the wild-type gene in colorectal epithelial cells with *APC* mutations results in apoptosis [146].

Given that methylation events have been reported very early on in colorectal tumourigenesis [37] and only APC can effectively initiate the neoplastic process in the colon [6], it is reasonable to assume that *APC* might be a target of deranged methylation. One investigation used bisulphite sequencing to characterize the methylation status of the 1A promoter (from which the major transcript is initiated) and found an exceedingly dense cancer-specific methylation, coupled with a loss of transcription [149]. The frequency of methylation was identical for small and large adenomas and adenocarcinomas (18%), absent from normal mucosa, and did not vary with Dukes' staging [149]. Methylation of *APC* is both an effective second hit in tumours with mutations in one allele and is responsible for the biallelic silencing of wild-type genes. Thus, it occupies the same, early position in the adenoma–carcinoma sequence as *APC* mutations and represents a change in temporal conformity with the CIMP+, bolstered by a significant predilection for adenocarcinomas of the caecum and ascending colon [150]. These data ought to be confirmed, though, given that another study found equally low levels of methylation in both normal mucosa and adenomas, but did not correlate this with expression [150].

The nature of the APC/β-catenin pathway suggests that the two are interchangeable lesions. However, the matchless gatekeeper status of APC is affirmed by Esteller *et al.* [149], who found that *APC* was hypermethylated at equal frequencies in colorectal tumours with either wild-type (11%) or mutant (14%) *β-catenin*. In conclusion, these findings further underscore the importance of *APC* inactivation in the initiation of colorectal tumours.

Cell-cycle regulators

The locus 9p21 provides an insight into the range of mechanisms that tumours exploit to silence anti-oncogenes [9]. This region is frequently subject to LOH and homozygous deletions in many cancer cell lines and primary tumours [151,152]. $p16^{INK4a}$ (also known as MTS-1 for multiple tumour suppressor, INK4a for inhibitor of cyclin-dependent kinase 4a, and CDKN2A for cyclin-dependent kinase inhibitor 2A) was originally described by Serrano *et al.* [153] and was later identified as the putative target of LOH on chromosome 9p [154]. It encodes a cell-cycle regulatory element that blocks progression through the G_1/S restriction point (between the first gap and DNA synthesis phases) [155]. $p16^{INK4a}$ binds to CDK4 or CDK6 and interferes with the formation of binary cyclin D–CDK4 complexes [155]. If production of the complex is unregulated, it goes on to phosphorylate the Rb (retinoblastoma susceptibility gene) protein and releases cells from their quiescent state [155]. Deletions or mutations in $p16^{INK4a}$ affect the balance between functional $p16^{INK4a}$ and cyclin D, resulting in abnormal cell cycling and growth [156]. Moreover, it appears that only one member of the cyclin D–Rb pathway has to be defective to confer this selective advantage [155]. By extension, the loss of function of one component occurs independently of, and is inversely related to, transformation of the others.

Consistently, allelic loss at the 9p locus and homozygous deletions of $p16^{INK4a}$ have not been found in CRA-derived cell lines or primary colorectal tumours [49,152,154]. Likewise, the Rb protein itself is normal [151,157] and coding-region mutations have not been reported in $p16^{INK4a}$ [158], yet $p16^{INK4a}$ expression cannot be detected in some cell lines [151]. In the absence of genetic causes, the focus turned to epigenetic lesions in the cyclin D–Rb pathway, in an attempt to define the tumour suppressor status of $p16^{INK4a}$ [9].

The earliest results from two independent groups were published in tandem [49,60]. Both reported a significant correlation between CGI methylation, in exon 1 of the $p16^{INK4a}$ gene, and its transcriptional silencing. However, their other findings were very different and controversial. One centre [60] communicated that in only 10% of primary tumours and colon cancer cell lines was the first exon of $p16^{INK4a}$ methylated, in comparison with 60% of adjacent normal colonic epithelium. Moreover, 50% of the tumours had raised expression of $p16^{INK4a}$, over and above that found in the normal epithelium. The evidence presented by the Johns Hopkins team [49] was more in keeping with scientific reasoning. Their data demonstrated hypermethylation at the 5′ CGI of $p16^{INK4a}$ in 92% of cell lines, 16% of adenomas, and 40% of primary tumours. All instances were correlated with a lack of expression of $p16^{INK4a}$, which could be restored in cell lines by deAZA treatment, concurrently with the restoration of growth control [49,74]. This implies that the aberrant DNA methylation is essential for the maintenance of transcriptional silencing. No hypermethylation was present in any of the normal colonic epithelium tested [49].

On cross-examination of their conflicting data, the authors propose several explanations [49,60]. A pre-publication cross-analysis of the contradictory samples ruled out methodological artifacts as the root of the discrepancy. The analysis of different CpG sites may be responsible [55], but one restriction site (*SacII*) was used by both groups. On consideration, the most likely reasons are that the mucosa-enriched samples of Gonzalez-Zulueta *et al.* [60] either contained

sub-populations of cells already harbouring abnormalities of *p16*^{INK4a} methylation [49], or comprised a mixed population of cells of different origins [159]. Alternatively, regional differences in the methylation status of the normal colonic mucosa may be responsible [68].

Further investigations, using more sensitive and reliable methods to detect methylation [10,58,73, 158–160], have helped to clarify the issue. The current consensus favours cancer-specific hypermethylation of *p16*^{INK4a}, with an incidence of *ca.* 28–55% in the colon [10,49,70,73,158–160]. This is bolstered by the colorectal cell line HCT116, in which the two *p16*^{INK4a} alleles are respectively inactivated by hypermethylation and a frameshift mutation [161]. On this basis, it is proposed that the tumour spectrum of *p16*^{INK4a} methylation is virtually the same as that for mutations, i.e. each event offers an equivalent growth advantage [71].

Detection of epigenetic *p16*^{INK4a} inactivation in transitional colonic mucosa and adenomas [10,49,159] and a lack of correlation with tumour stage [10,73,158] indicate that this is a common and early step in the adenoma–carcinoma sequence. The frequent detection of *p16*^{INK4a} hypermethylation in pre-neoplastic ulcerative colitis lesions broadens these findings to include other aetiologies [162]. The largest of the studies on *p16*^{INK4a} [73] also reported associations with female gender, a proximal location (i.e. of the caecum or ascending colon), and poor differentiation. Still larger studies, though, are needed to resolve conflicting evidence from a smaller series [158].

Initial confusion surrounding the status of *p16*^{INK4a} as a tumour suppressor gene launched a hunt for alternative targets of LOH at 9p21. Other resident genes at this locus include *p14*^{ARF} and *p15*^{INK4b}, which lie in very close proximity to *p16*^{INK4a}. Indeed, *p14*^{ARF} and *p16*^{INK4a} share an exon, albeit in a different reading frame [163]. p14^{ARF} induces G_1 and G_2 phase arrest through a p16^{INK4a}-independent mechanism [163] and it is believed to participate in a regulatory feedback loop with p53 and MDM2 [164]. The mouse homologue is also essential for the activation of p53, in response to oncogenic Ras [165]. p14^{ARF}'s role as a tumour suppressor gene in colorectal cancer was examined by Esteller *et al.* [160]. The incidence of *p14*^{ARF} hypermethylation was reported to be similar to that of *p16*^{INK4a}, with epigenetic inactivation occurring in approximately one-quarter of all primary CRAs and representing an early lesion in the adenoma–carcinoma pathway. However, the *p14*^{ARF} CGI demonstrates selective, epigenetic silencing in a subset of tumours, with a hypermethylated promoter between two unmethylated promoters (*p16*^{INK4a} and *p15*^{INK4b}), which are frequently methylated in other tumours. Moreover, hypermethylation of the 5′ CGI of *p15*^{INK4b} is not present in colorectal cell lines or primary CRAs [60,166]. This is probably because frameshifts in the polyadenine tract of the type II TGFβ receptor (as a result of microsatellite instability), abolition of the growth inhibitory effects of TGFβ by K-Ras [167], or loss of chromosome 18q [146] supplant the need to inactivate p15^{INK4b}, which regulates the TGFβ pathway [168].

The rich interplay of genetic and epigenetic events at this locus has provided an insight into the dysregulation of methylation in cancer. Theoretically, *p14*^{ARF} and *p16*^{INK4a} could be methylated as a downstream consequence of DNMT1 up-regulation, attributable to *Ras* mutations [117], but one study reported that methylation of *p16*^{INK4a} was not associated with K-*Ras* mutations [158]. However, conflicting evidence alleges that p16^{INK4a} expression is related to K-*Ras* mutational status in IEC-18-R_1 cells [159] and that CIMP+ tumours have a higher incidence of K-*Ras* mutations [78]. Another hypothesis implies that the inactivation of p16^{INK4a} and K-Ras could be mutually exclusive [169]. Colorectal tumour cells have absolute requirements for either mutations in *p53* or hypermethylation of the *p16*^{INK4a} promoter, to overcome Ras-induced senescence [169]. The overexpression of p53 does not co-segregate with *p16*^{INK4a} hypermethylation [158] and wild-type *p53* predominates in CIMP+ tumours [78], supporting this assumption. p16^{INK4a} and p53 play diverse roles from this response, though, and their co-existing inactivation may be necessary later in the adenoma–carcinoma series [78]. Furthermore, p14^{ARF} is linked to the status of both p53 and Ras, although neither shows a significant association with *p14*^{ARF} hypermethylation in preliminary data [160]. Mechanistic investigations are needed to give credence to one or other of these papers.

In summary, these epigenetic studies support a role for p16^{INK4a} as a tumour suppressor, governing cell progression through the G_1/S restriction point [156]. This locus provides a consummate example of the tissue and gene specificities of aberrant DNA methylation. Indeed, the approximation of so many tumour suppressor promoters may help to determine exactly how DNA methylation patterns are altered in cancer.

The mismatch repair apparatus

Deficiencies in the mismatch repair (MMR) system result in mutation rates 100-fold greater than normal cells, as a direct consequence of an inability to replicate the genome faithfully. In particular, these mutations are evident as frameshifts in microsatellite sequences (genetic loci consisting of 1–5 base pairs repeated 15–30 times). They are normally stable, but slippage during DNA replication generates insertions/deletions and, if perpetuated, engenders microsatellite instability (MSI), the hallmark of the replication error phenotype [170]. The defects in MMR first became apparent as germline mutations in the human *MutL* and *MutS* homologues *hMLH1* and *hMSH2* (and more rarely, *hMSH6*, *hPMS1*, and *hPMS2*) in HNPCC kindreds [171], which bestow an increased rate of progression on adenomatous polyps [6]. MSI is also present in *ca.* 10–15% of cases of sporadic colorectal cancer [170], albeit that mutations in MMR genes are rarely

detected in these non-hereditary cases [172]. This led to the supposition that other mechanisms are responsible for the defect [172].

Early work reported that different epigenetic phenotypes distinguish the mucosa in MSI+ and MSI− sporadic colorectal cancers [40]. This complements the hypothesis that the development of CRAs necessitates the acquisition of genetic instability by one of two pathways [6,40]. Gross chromosomal losses and/or gains of a large number of alleles are often present in cancers with functional MMR systems [40,63]. Alternatively, a defective MMR system (as a part of the CIMP) can lead to the accumulation of point mutations and small deletions and/or insertions in diploid cells [40,63]. Lengauer *et al.*'s association led them to suggest that genetic instability may be caused by either a CIMP-related MMR mutator phenotype [3], or a phenotype in which hypomethylation dysregulates chromosome segregation processes in cancer cells [173,174]. While a cancer's requirement for genetic instability stands up to scrutiny [77,175], it is not unchallenged [21] and recent work has refuted a mutual segregation of the hypermethylator and MSI+ phenotypes in colorectal cancer [10,63].

An alternative, but equally mistaken, hypothesis was later proposed [176,177]. Cytosine-rich strand hairpins in the fragile X syndrome were reported to serve as excellent substrates for DNMT1 [176], prompting some to propose that MMR defects themselves cause aberrant methylation [177]. The temporal and causal relationships between MSI and methylation were later resolved within the context of the CIMP [10]. First, *ca.* 50% of adenomas were classified as CIMP+, but *hMLH1* CGI methylation was not present in these lesions [10]. Second, *hMLH1* promoter methylation was absent from more than half of the CIMP+ cancers and from 25% of those with MSI [10]. Lesions in other MMR genes have not been reported in CIMP+ cases [178] and the hierarchy of CIMP and MSI is backed by successive studies [63,77].

A large amount of data has been amassed on *hMLH1* methylation, in order to clarify the nature of the above relationship. The CGI 5′ to *hMLH1* was originally described by Kane *et al.* [179] using Gardiner-Garden and Frommer's accepted definition [27]. The presence of cytosine methylation was analysed between nucleotides −67 and −670 of the promoter region (because of the high CpG density in this area), a protocol followed by all future investigators when examining *hMLH1* methylation with a variety of different methods [50,180–184]. Proof of a causal relationship was provided by cancer cell lines, in which the demethylator 5-azacytidine (AZA) was able to reactivate the gene and restore MMR proficiency [180]. On washout of the drug, the original pattern of methylation returned, verifying that this is an ongoing process [182]. Several of these studies, though, reported 'background' methylation of *hMLH1* in MSI− tumours and cell lines with MMR gene mutations [50,180,182]. Deng *et al.* [61] resolved this issue with a more detailed dissection of the *hMLH1* promoter region, which examined all the CpG sites concerned. The results demonstrated that only methylation in a region −248 to −175 from 'start' correlated with expression, which may explain the discrepancies discussed by Herman *et al.* [180]. In future, the methylation analysis should incorporate a breakdown of the CGI to delimit the relevant areas.

In spite of much conjecturing, *hMLH1* methylation in the CIMP is now believed to account for up to 75% of cases of sporadic CRAs with MSI [10]. This ties in well with the literature, which indicates that MMR gene mutations are identifiable in at least 26% of CRAs with MSI [180]. No study has been able to conclude whether or not methylation is a second hit in HNPCC [144,183,184]. In ulcerative colitis-associated neoplasia, MSI is found at an equal prevalence to sporadic cases and loss of expression of hMLH1 is not a frequent event [185]. However, 5′ CGI methylation of *hMLH1* has been reported in ~15% of inflammatory bowel disease-associated tumours [186].

Several tumour suppressor genes are downstream targets of MSI in colorectal cancer, for instance, the type II TGFβ receptor (a cell growth regulator with a polyadenine tract) [168] and BAX (a proponent of apoptosis with a tract of eight deoxyguanosines) [187]. Moreover, hMLH1 overexpression has been reported to trigger programmed cell death directly [188]. Thus, the 5′ methylation of *hMLH1* may influence cell growth, both directly and via its downstream targets.

Direct repair

O^6-methylguanine-DNA methyltransferase (MGMT), a ubiquitous DNA repair enzyme, transfers the alkyl group from DNA bases to a specific cysteine residue in the enzyme, in a suicide reaction [189]. Physiological donors for these lesions include dietary nitrates, reduced by bacterial flora in the proximal colon, and nitrosated amines/amides, from endogenous protein turnover [178]. If left unrepaired, O^6-methylguanine will mispair with thymine and undergo a guanine–cytosine (G–C) to adenine–thymine (A–T) transition on replication [190], or O^6-alkylguanine may cross-link, blocking DNA replication [190]. Consistent with this, *MGMT*-knockout mutants exhibit increased rates of mutations and cancers when exposed to ethylnitrosourea and other alkylating agents [189]. Nevertheless, the loss of expression of MGMT also limits the cytotoxic efficacy of *N*-alkyl *N*-nitrosurea-type chemotherapy drugs, e.g. 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU) [190].

The genoprotective effect of MGMT is lost in a subset (19–35%) of human tumours [191]; however, the gene is not commonly mutated, deleted or rearranged [192]. Methylation analysis of the 5′ *MGMT* CGI shows that dense methylation occurs at a frequency of 26–38% in colorectal cancer [192,193] and is significantly associated with reduced MGMT expression, which determines its catalytic activity [193]. More

extensive work, done previously on cell lines, found an association between two [100] or three [194] methylation 'hot spots' in the 5′ flanking region, silencing of the gene, and heterochromatinization of the transcription start site [195,196]. Expression could be re-established in these cells by treatment with AZA [100] or TSA [194], consolidating the causal nature of the relationship. Although a lack of exclusivity was documented between 5′ methylation and the loss of expression of MGMT [192], this may be mitigated by an early paper, which claims that *MGMT* methylation is not a molecular switch for transcription, but that it operates in a graded fashion [192].

As for mismatch repair, the large number of substrates for this mutagenic adduct offers a huge potential for genetic–epigenetic interactions. This is best illustrated by CRAs, which have a frequency of K-*Ras* mutations (40%) that approximates the frequency of 5′ *MGMT* methylation [178]. An association between MGMT expression [197] and guanine–cytosine to adenine–thymine transitions in K-*Ras* is highly significant ($p<0.01$). Likewise, *MGMT* methylation is also significantly associated with this particular genetic change ($p=0.002$) [198]. The reported ability of K-Ras to induce the expression of DNMT1 calls into question the cause or consequence nature of this correlation. However, the detection of errant *MGMT* methylation in small adenomas [192] precedes the appearance of K-*Ras* mutations [5]. Additionally, *MGMT* methylation is not associated with 5′ $p16^{INK4a}$ or $p14^{ARF}$ methylation [192], showing its independence as an epigenetic event.

In summary, aberrant methylation may shut down the expression of DNA repair genes, leaving the genome defenceless against exogenous and endogenous mutagens. The tumour may then accumulate mutations, which select it for progression towards an adenocarcinoma [178].

Cyclooxygenase-2

Contrary to the genes considered until now, several genes are consistently up-regulated, but not mutated, in colon cancer. Cyclooxygenase (COX)-2 is over-expressed in up to 43% of adenomas and 86% of carcinomas [198], and regulates angiogenesis [199], increases metastatic potential [200], and inhibits apoptosis [201]. Biochemically, the cyclooxygenases are the rate-limiting enzymes in eicosanoid (inflammatory mediator) synthesis from arachadonic acid. In contrast to the constitutively expressed *COX-1*, *COX-2* is a primary response gene, up-regulated by cytokines, growth factors, and tumour promoters [202]. Furthermore, COX-2 offers growth advantages to colorectal cancer cells and has significant early-stage tumourigenic properties [24].

Antithetically, *COX-2*'s promoter is also methylated in CIMP+ tumours, despite the apparent growth disadvantage. This may be reflected in a lower frequency of methylation (*ca.* 15% in adenomas and adenocarcinomas), compared with $p16^{INK4a}$ [49] and the cancer-specific MINT loci. However, the growth disadvantage is offset against a potentially increased resistance to treatment with non-steroidal anti-inflammatory drugs (see below) [203]. There is a significant inverse correlation between *p53* mutations and *COX-2* promoter methylation [76], in accord with the ability of wild-type p53 to suppress *COX-2* expression [204]. This may have direct implications for patient treatment (see below).

Angiogenesis/metastasis-related genes

The 5′ promoters of *THBS1* [10,205] and the gene encoding tissue inhibitor of metalloproteinase III (*TIMP3*) [206] are methylated in 24% and 28% of CRAs, respectively. *THBS1* encodes an anti-angiogenesis factor with tumour suppressor properties, which is positively regulated by p53 and Rb [205]. Its loss of expression may therefore aid the neovascularization of the tumour and its haematogenous metastasis. Furthermore, methylation of the *THBS1* promoter, as part of the CIMP, has been correlated with the presence of wild-type *p53*, but this may be due to a positive association with $p16^{INK4a}$ and *COX-2* expression [10].

TIMP3 is an extracellular matrix binding protein, which may be necessary to suppress tissue remodelling, angiogenesis, invasion, and metastasis [206]. By due accord, methylated alleles of *TIMP3* and *THBS1* are found in invasive adenocarcinomas, although little data exists on their status in pre-malignant cells [10,205,206]. Taken together, tumours that repress expression of THBS1 and/or TIMP3 (such as those with the CIMP defect) may be more aggressive. Additional studies are called for to test this hypothesis.

The Peutz–Jeghers' susceptibility gene

Linkage studies done on Peutz–Jeghers' syndrome (PJS) kindreds and allelotyping of PJS adenomas and adenocarcinomas identified a germline mutation in a gene at chromosome 19p13.3 in six families [207]. LKB1/STK11 is a serine-threonine kinase which acts as an early gatekeeper, regulating the development of hamartomas in PJS. These may be the pathogenic precursors of adenocarcinomas in PJS patients [207]. By pursuing a similar line of investigation that was discussed for HNPCC and FAP, the *LKB1/STK11* gene was found to be epigenetically silenced in both primary tumours associated with PJS (18% of hamartomous polyps) [208] and 8% of sporadic colorectal cancers [209]. Such a low frequency in sporadic adenocarcinomas is in keeping with the literature, which supports APC, not LKB1/STK11, as the principal gatekeeper in the colonic epithelium [5,209].

Genes not epigenetically inactivated in colorectal cancer

The involvement of non-tumourigenic genes (e.g. *CALCA*) is typical of a model whereby the pattern of

gene inactivation is determined not by their function, but by the structural properties of the genome. This information can be used to discern patterns of methylation in ageing and cancer and it may help to identify experimental controls [55]. However, given the current publication bias towards positive data, there are only a few reliable studies in the literature, for instance, *14-3-3* σ [66], mismatch repair genes other than *hMLH1* [178], glutathione *S*-transferase π1 (*GSTP1*) [210], the juvenile polyposis syndrome susceptibility gene *SMAD4* [211], and *Fas* [212]. The silencing of other genes by promoter methylation has not been resolved, e.g. the Wilms' tumour gene (*WT1*) in the colorectal epithelium [213]. Given that many of the promoter CGIs mentioned here are hypermethylated in other cancers, there is a strong argument that cancer epigenetics, like genetics, is a tissue-specific event [9].

A place for epigenetics in colorectal tumourigenesis

Some of the earliest events in colorectal dysplasia are epigenetic. To illustrate, both the extent and the patterns of methylation are altered in adenomatous polyps [37], but this can be interpreted in many ways. For instance, it is possible that the CIMP+ colonies could have originated from stem cells that had different patterns of methylation from the bulk of the normal, differentiated cells used for comparison [40]. Only characterization of the elusive stem cells can provide the answers. Until then, terms such as 'hypomethylation' and 'hypermethylation' should be viewed with caution [40]. In addition, the characterization of a cancer's epigenetic profile must contend with evolution, which may take multiple cell cycles for sufficient density of methylation to accumulate and correlate with maximal silencing of expression [127]. In spite of these problems, the articles reviewed herein endorse the provisional modification of Vogelstein's genetic model to include promoter-region CpG methylation as a significant, early event in tumourigenesis.

Applications

Implications for oncologists

Despite evidence that adjuvant chemotherapy with 5-fluorouracil (5-FU) improves disease-free survival and overall survival in colorectal cancer patients, there are several controversies concerning its application [214]. It has long been recognized that distinct subsets of sporadic colorectal cancer exist (e.g. the MSI phenotype); however, these genetic data have not been fully exploited to optimize specific treatment regimens [215]. CIMP+ tumours may represent one phenotype, which could be targeted by demethylating agents. Furthermore, the idiosyncratic fingerprint of epigenetic and genetic events may specifically cultivate resistance in these tumours to other chemotherapeutic agents.

Methylation, like most enzymatic processes, has been measured [59] and can be slowed and possibly reversed, using appropriately targeted interventions [68]. The DNA methyltransferase antagonist AZA and its deoxy analogue, deAZA/decitabine, are potent demethylating agents [215]. AZA is believed to form a covalent complex with DNMT1, allowing DNA replication to occur in the absence of methylation [134], with subsequent demethylation of the colony. DNMT1 activity is normally directed to specific sequences and may be amenable to pharmacological intervention that will result in an organized change in gene expression [134]. However, the non-specificity of these two drugs does not reflect this. AZA can induce differentiation in *Aspergillus* [134], which does not contain 5-methyl-cytosine in its genome [216], and is a potent mutagen at mammalian CpG dinucleotides [216]. This latter effect is mediated by the interaction of AZA with DNMT1 as it binds to DNA, which forms an adduct with DNA-damaging properties [216]. Originally synthesized as a pyramidine analogue for anti-cancer chemotherapy [134], AZA is consequently cytotoxic and inhibits tRNA methylation, protein synthesis, DNA replication, and the enzymes affiliated with these processes [134,215].

In spite of these misgivings, oncologists have proceeded with phase II clinical trials in haematopoietic tumours [217,218], which are ideal for the temporal monitoring of treatment [127]. To date, deAZA has shown promise in some cases, but toxicity and clinical resistance have been observed [134]. Moreover, no study has demonstrated a relationship between clinical efficacy and target gene demethylation, although deAZA can overcome drug resistance in human tumour xenografts [219] and may be used as an adjunct to mainstream chemotherapy. A proper evaluation of the efficacy of this approach depends on the development of agents that block DNMT1 before its interaction with DNA. The delivery of antisense molecules targeted against DNMT1 mRNA has been studied *in vivo* [113,114], but their potential to inhibit tumourigenesis has yet to overcome the many obstacles facing gene therapy. The application of gene therapy itself may also be hampered in CIMP+ tumours, which could feasibly methylate and silence exogenous transgenes [36]. Another course might see oncologists target other epigenetic phenomena associated with DNA methylation, such as histone deacetylation. Once hyperacetylation has been induced by apicidin (an HDAC inhibitor), the cells undergo selective changes in the expression of $p21^{WAF1}$ and gelosin, which control the cell cycle and cell morphology [220]. Evidence for a corresponding cell-cycle arrest in the G_1 phase warrants further investigations of apicidin's antiproliferative efficacy. This line of investigation should clarify whether the reported dominance of DNA methylation, over histone deacetylation, in cell lines [96] is mirrored *in vivo*.

Treatment regimens have also been guided by the epigenetic and genetic constitution of CIMP+ cancers.

For instance, promoter methylation of *MGMT* may render tumours susceptible to alkylating agents, e.g. carmustine [221]. The groundwork for this hypothesis has been performed on gliomas, which have a similar frequency of *MGMT* methylation (40%) to CRAs [192]. Esteller *et al.* [221] found a highly significant correlation of *MGMT* promoter methylation with a partial or complete response to carmustine. In gliomas, MGMT activity was the only predictor of survival; a lack of methylation had a hazard ratio of 9.5 for the risk of death [209]. If these data are reproducible in colorectal cancer, it could lead to the re-appraisal of chemotherapy regimens, based on the tumour's molecular profile. In other cancers which express MGMT, the use of an enzyme inhibitor, such as O^6-benzyl-guanine, may overcome any resistance to alkylating agents and limit the toxic effects of carmustine [221–223]. Invariably, though, MGMT-deficient cancer cells are also resistant to O^6-benzyl-guanine [224].

Given that MMR deficiency is strongly, but not exclusively, associated with the CIMP [10], its therapeutic implications are also relevant. An intact MMR system can recognize 5-FU incorporated into DNA (a side-effect of its accumulation) and direct the cell towards apoptosis [225]. However, in cell lines with MMR defects (such as those with *hMLH1* promoter methylation), the 5-FU lesions are not detected and the tumour is resistant *in vitro* [225]. Likewise, a publication from the Johns Hopkins group found that p53 disruption rendered cells strikingly resistant to the effects of the 5-FU anti-metabolite [226]. However, CIMP+ tumours, at least in the early stages of colon cancer, are associated with a wild-type *p53* [78]. A definitive answer to this quandary awaits the characterization of the CIMP's molecular profile and a large prospective clinical trial of 5-FU in this subgroup. In the meantime, an investigation into Dukes' stage C colon carcinomas has reported that the presence of a p53 mutation did not predict for survival, but that MSI in the proximal/transverse colon carcinoma group was associated with a 25% lower 5-year mortality ($p=0.015$) [227]. Thus, CIMP+ cancers may represent a clinically responsive subgroup.

Of the other epigenetically inactivated genes, a paucity of COX-2 expression may attenuate the tumour's response to growth suppression by celecoxib (a specific COX-2 inhibitor) [228] or NSAIDs such as sulindac [203]. The use of p53 and K-Ras as novel indicators of tumour aggressiveness in colorectal cancer [229] could be applied to the grading of CIMP+ tumours, but the current consensus precludes their use in routine prognostic evaluations [230]. Further, the prognostic significance of CDK inhibitors (for instance, $p16^{INK4a}$) remains uncertain in colorectal cancer [158,231,232].

More work is necessary in this area, but these drug–gene interactions could conceivably have an impact on medical practice.

Inflammatory bowel disease

Both the 'normal' mucosa and dysplastic lesions associated with inflammatory bowel disease and its sequelae display epigenetic transitions reminiscent of those seen in cancer [162,186]. The epigenetic changes in these conditions are strongly influenced by certain therapies [233], which may be more widely applicable to CRA therapy as a whole. Glucocorticoids modify chromatin by binding CBP, a transcription co-activator that acetylates core histones [234]. Methotrexate is hypothesized to modulate DNA hypomethylation in inflammatory arthritis, but there are discrepancies and its mode of action remains undetermined [235]. A fermentation product of gut bacteria, butyrate, selectively induces $p16^{INK4a}$ expression, growth arrest, and differentiation in colon cancer cells [236], apparently by acetylation of H4 [237]. This symbiotic compound could help to maintain the genomic integrity of the epithelium; in clinical trials, butyrate has proven to be an effective primary and/or adjunctive therapy in some patients with ulcerative colitis [238].

Cloning new tumour suppressor genes

Classical positional approaches have allowed the cloning of genes such as *Rb* and *APC*. However, in a novel approach, Wales *et al.* [12] followed up studies showing that the 17p13 locus is a frequent site of LOH and contains a CGI, which is aberrantly methylated in many human cancers. The combined use of RNase protection strategies, exon trapping studies, and northern blotting identified the gene, hypermethylated in cancer (*HIC*)-1 [12]. Their success is supported by evidence that *HIC-1* is a strong candidate for a tumour suppressor gene, since its expression is regulated by wild-type *p53* [12]. At the time, this was unique, but soon a fresh hunt for tumour suppressors such as HIC-1 was heralded by techniques such as methylated-CGI amplification (MCA) [57] and restriction landmark genome scanning (RLGS), which can rapidly disclose the methylation profiles of thousands of genes in parallel [59]. For instance, soon after they employed MCA to define the CIMP, Toyota *et al.* [57] used the MCA fragments to probe cDNA libraries. By correlating the loci of the MINT CGIs with regions of LOH, MINT31 was identified as a potential marker for a tumour suppressor gene. Genomic sequencing of the area 17q21, frequently lost in colorectal cancer [239], revealed a T-type calcium channel (CACNA1G) later found to be 5′ methylated in 35% of colorectal cancers [240]. The exact role of this calcium channel in cancer has not been settled, but it may modulate calcium signalling and potentially affect cell proliferation and apoptosis [241].

Cancer research has focused on only a select few of the 45 000 CGIs in the human genome [242] and RLGS has thus been applied to broaden the scope of investigation [59]. One group has described the methylation status of 1181 selected CGIs across a

range of cancer types, guiding them to predict that an average of 608 CGIs are hypermethylated across the complement of tumour samples, although each neoplasm had a distinct methylation profile [59]. Their results are similar in low-grade tumours in which genetic abnormalities are rarer, with implications for the position of epigenetics in the adenoma–carcinoma sequence. The relationship of some of these CGIs with pertinent promoters and transcriptional activity suggests that the number of tumour suppressor genes has been grossly misjudged, due in part to a long-standing genetic bias in cancer research [59].

In the future, it is likely that these methods will be aided by a recently established methylation databank [243] (available from http://www.methdb.de), which integrates the available information on differentially methylated CGI clones. This will serve as a bridge between the well-publicized human genome project and its practical use for the cloning of novel tumour suppressor genes [11].

Tumour biomarkers

The use of CpG hypermethylation events as tumour biomarkers is more auspicious. Biomarkers can provide indices for early diagnosis, the detection of recurrence, cancer risk-assessment, and prognosis [244]. Methylated CGIs are associated with virtually every type of tumour [59], may be grouped into tumour-specific marker panels [59], are always modified in a defined region (unlike mutations, for example in *APC*) [18], and can be detected with a high degree of sensitivity using MSP [58]. These features make them ideal biomarkers [127].

The most sensitive MSP to date can detect methylated DNA in sputum, from patients with squamous cell lung carcinoma [245]. A sensitivity of up to 100% has been achieved by assessing both $p16^{INK4a}$ and *MGMT* promoter methylation [245]. The authors claim that aberrant methylation can be detected up to 3 years prior to a clinical diagnosis and may therefore significantly improve patient survival [245]. Moreover, these type C gene promoters are hypermethylated in DNA from cancer-free, high-risk subjects, approximating their lifetime risk for lung cancer [245].

In colon cancer, the 5′ methylation of *hMLH1* has been detected in the serum of 33% of patients with MSI– cancers [246]. The technique is 100% specific, but the small number of MSI+ cases ($n=9$) precludes any firm conclusions [246]. The loss of imprinting in tumours and tumour DNA in serum has been proffered as a biomarker for the diagnosis of colorectal cancer, but again more numbers are required [247,248]. Likewise, a larger series of cases is needed to evaluate the application of serological biomarkers, including CGI methylation, in the detection of metastatic spread from colorectal adenocarcinomas [249].

The technology already exists to screen for these biomarkers on a large scale. MCA may be applicable to automation for population-based analyses of putative epigenetic biomarkers [59] and real-time MSP can provide the necessary quantitative data for patient follow-up [62]. Nonetheless, critical issues still need to be resolved, particularly the point in tumourigenesis at which DNA in peripheral samples reaches the threshold for the positive detection of methylation changes. Direct comparison with other screening methods awaits much larger trials that fulfil strict criteria.

Concluding remarks

Ten years on from the discovery of hypermethylation-induced repression of *Rb*, epigenetics has been substantiated as a marker for neoplastic growth and may form the basis of a malignant phenotype. It would now be naïve to assume that alterations in the genetic code solely account for the pathogenesis of most tumours [9]. Large-scale epidemiological studies are still required for absolute confirmation of a CIMP, but prior and ensuing publications, from several groups, all support its existence. The clustering of epigenetic events in the CIMP may be likened to suddenly opening Pandora's box. The acquisition of an unknown defect in the physiological methylation machinery unleashes numerous molecular incidents, resulting in an acutely transforming phenotype [11]. While the exact position of this defect within the colorectal adenoma to adenocarcinoma sequence has yet to be determined, the methylation of the enterocyte gatekeeper, APC, suggests that it may be an early lesion. In particular, the APC/β-catenin, senescence, apoptosis, and TGFβ pathways are all inactivated in this subset of colorectal tumours [71]. Furthermore, there probably exists an uncharacterized pathway which is altered in 'normal' cells as a function of age-related methylation. Morphologically this is apparent as an expansion of the proliferative zone [71].

The nature of the initiating event in the CIMP has not been defined, but new findings in the highly conserved methylation machinery of plants show parallels to the human system [136]. Currently, the *trans*-acting factor theorized to set mammalian methylation patterns features largely in the hypotheses of many authors and is sought as the methylation defect. The ensuing epigenetic and genetic instability generates the molecular diversity that accompanies the clonal evolution of neoplasia [175]. Thus, in view of the complex interdigitation of genetic and epigenetic events, the term 'epi-mutator phenotype' (after Loeb's mutator phenotype) [3] best describes cancers with a clustering of epigenetic events. Indeed, designating these tumours as positive for the 'CpG island methylator phenotype' is misleading, as it refers only to the epigenetic features of the cells.

The mapping of hundreds to thousands of CGI-associated tumour suppressor genes will (in the words of Eng, Herman, and Baylin) [250] 'keep cancer biologists off the streets for years to come.' Moreover, the discovery of the repressive properties of methylation

has inspired new areas of research into risk assessment, disease prevention, and treatment [67]. Future studies may bring more effective treatments for patients. In conclusion, scrutiny of the lately defined 'methylome' [251] holds significant heuristic potential and may provide colorectal researchers with new aetiological insights and clinical applications.

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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

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Purpose: **The impact of azacytidine (Aza C) on the quality of life of 191 patients with myelodysplastic syndrome was assessed in a phase III Cancer and Leukemia Group B trial (9221).**

Patients and Methods: **One hundred ninety-one patients (mean age, 67.5 years; 69% male) were randomized to receive either Aza C (75 mg/m^2 subcutaneous for 7 days every 4 weeks) or supportive care, with supportive care patients crossing over to Aza C upon disease progression. Quality of life was assessed by centrally conducted telephone interviews at baseline and days 50, 106, and 182. Overall quality of life, psychological state, and social functioning were assessed by the European Organization for Research and Treatment of Cancer (EORTC) Quality of Life Questionnaire C30 and the Mental Health Inventory (MHI).**

Results: **Patients on the Aza C arm experienced significantly greater improvement in fatigue (EORTC, $P = .001$), dyspnea (EORTC, $P = .0014$), physical functioning (EORTC, $P = .0002$), positive affect (MHI, $P = .0077$), and psychological distress (MHI, $P = .015$) over the course of the study period than those in the supportive care arm. Particularly striking were improvements in fatigue and psychological state (MHI) in patients treated with Aza C compared with those receiving supportive care for patients who remained on study through at least day 106, corresponding to four cycles of Aza C. Significant differences between the two groups in quality of life were maintained even after controlling for the number of RBC transfusions.**

Conclusion: **Improved quality of life for patients treated with Aza C coupled with significantly greater treatment response and delayed time to transformation to acute myeloid leukemia or death compared with patients on supportive care ($P < .001$) establishes Aza C as an important treatment option for myelodysplastic syndrome.**

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THE PROGNOSIS FOR patients with myelodysplastic syndrome (MDS) is grim, with an overall median survival for those with high-risk MDS ranging between 6 and 12 months.[1] Presently, no treatment has been proven effective, including antileukemia chemotherapy, hormonal therapy, and differentiation-inducing agents.[2,3] Allogeneic bone marrow transplantation (BMT) has offered the only real opportunity for cure, but because of treatment toxicity and the older age of the MDS population, it is an option for only a few individuals.[1,3]

In 1985, a new agent, azacytidine (Aza C) was tested for safety and efficacy in two phase II studies in patients with poor-risk MDS within the Cancer and Leukemia Group B (CALGB).[2] There was a demonstrated treatment response in 49% of 43 assessable patients (12% in complete remission; 25%, partial remission; and 12%, improved), with an overall median survival of 13.3 months. Transfusion requirements were eliminated in 82% (14 of 17) of patients who responded and had previously required RBC transfusions at study entry.[2] In the second study, Aza C was administered subcutaneously, with comparable results.[4]

On the basis of these findings, a phase III randomized trial was initiated in the CALGB (CALGB 9221) in 1993 to test the clinical efficacy of Aza C and its impact on quality of life.[5] It was hypothesized that a response to Aza C would result in improved quality of life attributable to better palliation, with less fatigue resulting in improved physical and social functioning and less psychological distress.

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PATIENTS AND METHODS

Research Procedures

All participants in the quality-of-life component of the clinical trial, CALGB 9221, had a diagnosis of MDS and on informed consent had been randomized to either Aza C (75 mg/m^2 for 7 days subcutaneously every 28 days) or supportive care.[5] Treatment arms were stratified by histologic subtype using French-American-British (FAB) criteria. Patients in both arms continued to receive best supportive care with transfusions, antibiotics, and hospitalizations. Eligibility requirements for the clinical trial were 16 years of age or older, an Eastern Cooperative Oncology Group (ECOG) performance status of 0 to 2,[6] and no other serious medical or psychiatric illness. After a minimum period of 4 months, those on the supportive care arm could cross over to the Aza C arm based on strict criteria concerning disease progression (see the study by Silverman et al in this issue of the *Journal of Clinical Oncology* for an extended description). Patients exited from the supportive care arm within the first 4 months only because of leukemic transformation or platelets less than 20×10^9/L.

Quality-of-life assessments were scheduled at the following times: study entry, before randomization; day 50 (corresponding to completing two cycles of Aza C, and 6 days before a bone marrow test to assess treatment response); day 106 (corresponding to completing four cycles of Aza C, and 7 days before re-evaluation of treatment response); and day 182, approximately 6 months after entry to the study, to capture any sustained quality-of-life benefits at the time of maximum treatment response, based on our previous experience.[2] Patients who crossed over from the supportive care arm to Aza C began the series of quality-of-life assessments again at that point. Quality-of-life assessment was discontinued when patients treated with Aza C either progressed or withdrew from the study.

Before randomization, patients were given a quality-of-life packet of measures on entry onto the clinical trial, with a request to complete it at home within 2 to 3 days. This was followed by a telephone interview generally lasting 30 to 40 minutes, conducted by two trained nurse research interviewers (E.P.D. and R.O.R). This procedure was repeated at all subsequent interviews, with the quality-of-life questionnaire packet mailed to patients 7 to 10 days before the scheduled interview. The use of centralized telephone interviews to collect quality-of-life data has been successfully used in numerous studies within the CALGB.[7]

Measures

The quality-of-life assessment consisted of standardized measures assessing patients' report of their physical symptoms and functioning, psychological state, and social functioning. All measures were administered at each assessment, except for sociodemographic questions, which were asked only at study entry, and the Perception of Improvement of Condition item, which was administered only to those taking Aza C at follow-up assessments.

European Organization for Research and Treatment of Cancer Quality of Life Questionnaire C30. The European Organization for Research and Treatment of Cancer (EORTC) Quality of Life Questionnaire-C30 is a measure of quality of life applicable to patients with any cancer diagnosis, consisting of 30 items concerning general physical symptoms, physical functioning, fatigue and malaise, and social and emotional functioning.[8,9] All subscale scores are transformed to a 0 to 100 scale. Higher scores on functional scales represented a better level of functioning; higher scores on symptom scales represented worsening symptoms.

Mental Health Inventory. The Mental Health Inventory (MHI),[10] a measure of psychological state, consists of 38 items grouped into the following five subscales: anxiety, depression, positive affect, emotional ties, and loss of behavioral and emotional control. The total score, the MHI index, and two global subscale scores, psychological distress and psychological well-being, are created from these subscales. Higher scores on the MHI index and subscales measuring positive affect and well-being indicate a better emotional state; higher scores on negative psychological states indicate a worse emotional state. The MHI has been tested on 5,000 respondents from six communities, which has served as the basis for the norms for the measure.[10]

Patients' perception of improvement in their condition. Patients randomized to the Aza C arm and those who later crossed over to treatment were asked at each follow-up assessment to rate whether they felt their condition was improving as a result of their treatment on an 11-point visual analog scale, from 0, "not at all," to 10, "complete improvement."

Sociodemographic and medical characteristics. Standard questions were used to obtain sociodemographic information at the time of interview,[11] and age and ethnicity were obtained at the time of patient registration. Medical information was collected from the medical record, including histologic diagnosis (as determined by central pathology review), ECOG performance status rating[6] at baseline, treatment response, and number of RBCs, platelet transfusions, and infections.

Statistical Considerations

Because of patient attrition over the course of the study attributable to disease progression, illness, and death, a pattern mixture model was used to analyze changes in quality of life over the study period, which took into account the number of quality-of-life assessments over time (ie, the pattern).[12,13] Patients were therefore categorized into four subgroups, based on the time of their last quality-of-life assessment, with subgroups generally coinciding with the number of assessments, as follows: subgroup 1, patients at study entry within 39 days after randomization, including a few patients with two assessments within this time interval; subgroup 2, mostly consisting of those assessed twice, with the last assessment occurring between days 40 and 82; subgroup 3, mostly consisting of patients assessed three times, with the last assessment conducted between days 83 and 159; and subgroup 4, mostly consisting of those assessed four times, with the last assessment conducted between days 160 and 259. These subgroups thus formed the patterns for the original two-arm design of the study. Within each subgroup, or pattern, a form of regression analysis, the linear random coefficient model, was used to test the effect of treatment arm and time on patients' quality of life. To statistically control for covariables to test the effect of crossing over to Aza C from supportive care on patients' quality of life, FAB subtypes and time elapsed after crossing over from the supportive care arm to Aza C were incorporated into the model. Data from subgroup 1 ($n = 31$) and subgroup 2 ($n = 18$) were combined in the analyses because of relatively small numbers in these subgroups and the assumption that treatment differences would be similar. For patients in the supportive care arm who crossed over to Aza C, subgroup classification was determined by the time from study entry to their last quality-of-life assessment on Aza C. It should be noted that although the original ideal points of assessment were study entry and days 50, 106, and 182, there was significant variability in the actual time patients were

assessed because of their illness or their being on vacation, delays in the mail, and interviewer and patient availability.

All of the quality-of-life measures were tested for significant differences between the treatment groups at baseline. The EORTC role functioning subscale was the only variable found to be significantly different at baseline between the two treatment arms. There were no other statistically significant differences between the two arms of the study for any of the remaining scales and subscales.

MHI scores were compared with norms for each of the subscales and total score to identify patients in severe distress with scores 1.5 SD above the norm.[10] Based on reports by Osoba et al[14] and King[15] that a 10-point change on the EORTC was comparable to a clinically significant improvement, the percentage of patients was calculated by treatment arm whose scores on EORTC subscales and total scores improved by 10 points or more at follow-up assessments from study entry levels.

Statistical Power Considerations

With 191 patients in the trial, the study had 80% power to detect a medium effect size of 0.57 (comparable with 0.54 of a SD, based on standardized means) between treatment arms in three quality-of-life measures for the change from baseline to the second follow-up assessment at day 106. Because there were three primary end points, MHI, EORTC fatigue subscale, and EORTC physical functioning subscale, the significance level used for determining the sample size was reduced to 0.017 (0.05/3) using Bonferroni's method.[16]

RESULTS

Patient Characteristics

One hundred ninety-one patients were accrued to the clinical trial from February 1994 to April 1996, with 99% having completed the baseline quality-of-life assessment. The mean age of patients was 67.5 years (SD, 10.3 years), and most were male (69%), white (93%), married (61%), and not presently employed (retired, 36%; disabled or unemployed, 23%) (Table 1). Our sample was more heavily represented by men than women than is usually indicated in epidemiologic studies.[1] There were no significant differences between the two treatment arms on study entry in any of the sociodemographic or medical characteristics.

Of the 99 patients initially randomized to Aza C, 56% (n = 56) remained on active treatment by day 182; 16% (n = 16) had died; 22% (n = 22) had terminated protocol treatment because of treatment failure, toxicity, or transformation to AML; and 5% (n = 5) refused to complete the quality-of-life questionnaires. Of the 92 patients initially randomized to supportive care, 47% (n = 43) remained on study with quality-of-life data collected through day 182, including 13% (n = 12) who remained on supportive care, 34% (n = 31) who remained on Aza C after cross-over, 23% (n = 21) who had died, 26% (n = 24) who had terminated protocol treatment, and 4% (n = 4) who refused to continue in the quality-of-life study. There were 80.4% (n = 74) and 61.9% (n = 57) of supportive care patients still on supportive care, completing quality-of-life assessments, at days 50 and 106, respectively.

Table 1. Patients' Medical and Sociodemographic Characteristics

Characteristic	Supportive Care (n = 92) (%)	Aza C (n = 99) (%)	Total (n = 191) (%)
Sex			
Male	65	73	69
Female	35	27	31
Race			
White	92	94	93
Black	5	3	4
Hispanic/Asian	2	3	3
Age			
30-49	5	7	6
50-59	13	10	12
60-69	40	37	39
70-79	27	36	32
80+	14	9	12
Mean ± SD, years	67.9 ± 10.3	67.3 ± 10.4	67.5 ± 10.3
Median, years	67	69	68
Range, years	35-88	31-92	31-92
Marital Status			
Married	65	58	61
Separated/divorced	5	6	6
Widowed	12	11	12
Single, never married	3	4	4
Unknown	14	21	18
Education			
1-11 grades	16	19	18
High school graduate	27	18	23
Some college/junior college degree	23	21	22
Bachelor's degree or higher	16	21	18
Unknown	18	20	19
Present employment			
Part- or full-time	24	17	20
Homemaker	3	3	3
Retired	36	35	36
Disabled/unemployed	21	24	23
Unknown	16	20	18
Performance status			
0	30	37	34
1	52	47	50
2	15	12	14
Unknown	2	3	3
Histology			
RA	20	19	19
RARS	3	4	4
RAEB	42	42	42
RAEB-T	20	22	21
CMMoL	7	6	6
Other	9	6	7

Abbreviations: RA, refractory anemia; RARS, refractory anemia with ringed sideroblasts; RAEB, refractory anemia with excess blasts; RAEB-T, refractory anemia with excess blasts in transformation to leukemia; CMMoL, chronic myelomonocytic leukemia.

Comparison of Quality of Life of Patients on Aza C Arm Versus Supportive Care Arm

Over time, patients on the Aza C arm experienced significantly greater improvement in fatigue (EORTC, *P*

= .001), dyspnea (EORTC, P = .0014), physical functioning (EORTC, P = .0002), positive affect (MHI, P = .0077), and psychological distress (MHI, P = .015) than those in the supportive care arm (Tables 2, 3, and 4). As can be seen in Figs 1 through 4, which illustrate the EORTC fatigue, dyspnea, physical functioning, and MHI psychological well-being subscales, patients' quality of life for subgroups 3 and 4 was generally stable or worsening while on supportive care, compared with an improving quality of life for those on the Aza C arm. This was statistically demonstrated by the slopes of the regression lines for subgroups 3 and 4 often being in the opposite direction for the Aza C arm compared with the supportive care arm for many of the measures (Table 4 and Figs 1 through 4). Despite the considerable variability in patients' reporting of symptoms and functioning, as seen by the standard errors of the slopes in the regressions (Table 4), many of the differences between treatment arms were highly significant.

The correlations between the baseline measures of physical symptoms and functioning (ECOG performance status; EORTC subscales) with those of psychological state indicated a significant interrelationship between patients' physical status and psychosocial state (MHI psychological distress, r = .17 to .46; P < .05 to < .0001; median, r = .30; P < .001). These correlations suggested that physical improvement was the likely cause for the psychological improvement of patients taking Aza C.

Because RBC transfusions were more frequently administered in the Aza C group compared with the supportive care arm (P = .002) during the first month on study, it was possible that these transfusions were responsible for the significant improvements in patients' fatigue, physical functioning, and psychological state rather than Aza C. When RBC transfusions were statistically controlled for in the linear random coefficient model, significant differences between treatment arms were still maintained at the adjusted alpha level for the EORTC fatigue, dyspnea, physical functioning, and MHI psychological well-being subscales. The sole exception was the MHI psychological distress subscale, with treatment arm differences becoming nonsignificant at P = .017 (Bonferroni-adjusted alpha level),[14] on controlling for RBC transfusions (P = .038).

Quality-of-life measures before and after cross-over were compared for patients who had at least one quality-of-life assessment after cross-over (n = 38). Before cross-over, patients' quality of life was found to be either stable or slowly worsening, varying by quality-of-life area. However, subsequent to cross-over to Aza C, there was a significant improvement in the rates of change in several areas, comparable with those observed for the entire sample, including EORTC physical functioning, fatigue, dyspnea, and overall quality-of-life subscales and MHI global psychological distress and well-being subscales at the adjusted alpha level (Table 4). Figures 5 and 6, concerning several EORTC and MHI subscales, portray this for the 30 supportive care patients who crossed over to Aza C after approximately 4 months on supportive care and who were followed for a mean of 4 months on Aza C therapy. Patients also reported that their conditions were improving after cross-over to Aza C (P = .0001).

FAB histology subtypes were grouped into patients with better (refractory anemia [RA], RA with ringed sideroblasts; n = 44) and worse (RA with excess blasts, RA with excessive blasts in transformation to leukemia, chronic myelomonocytic leukemia; n = 133) prognoses[1] to test whether disease prognosis influenced the relationship between treatment arm and quality of life. There was no evidence of histologic subtypes significantly influencing patients' quality-of-life scores.

Clinical Significance

The ECOG performance status and MHI psychological distress subscale were used to examine whether the statistically significant differences in psychological status and physical functioning between treatment arms were clinically meaningful. The translation of the EORTC physical functioning scores to ECOG ratings at follow-up assessment from baseline levels was done by calculating the means of the EORTC physical functioning scale scores at baseline for the total sample for each of the ECOG ratings (ECOG 0 = 74.9; 1 = 62.7; 2 = 38.3). These scores then served as benchmarks for each of the ECOG ratings. The benchmark EORTC physical functioning means at baseline for ECOG 0, 1, and 2 were then subdivided into deciles matched to deciles we constructed between ECOG ratings of 0 and 1 and 1 and 2. The actual EORTC scores for each of the subgroups for each treatment arm were then compared with these benchmark EORTC decile means related to the decile ECOG ratings.

Using these values as benchmarks for a clinically meaningful change, the improvement in physical functioning in Aza C subgroup 3 patients at day 106 was comparable with an improvement in the ECOG score from approximately 1.3 to 0.7, whereas patients in the supportive care arm worsened from an ECOG status of 1.1 to 1.5 (see Table 5). Analyses of subgroup 4 were somewhat similar, with Aza C patients showing that an improved EORTC physical functioning score at day 182

Table 2. Means of Selected EORTC OLQ-C30 and MHI Scales for Each Subgroup for Supportive Care*

	Supportive Care											
	Baseline			F/U 1			F/U 2			F/U 3		
QoL Scale	Mean	No.	SD	Mean	No.	SD	Mean	No.	SD	Mean	No.	SD
EORTC												
Physical functioning†												
Subgroup 1	52.0	15	29.1	—	—	—	—	—	—	—	—	—
Subgroup 2	77.8	9	18.6	56.0	10	32.4	—	—	—	—	—	—
Subgroup 3	63.5	23	28.7	62.7	22	32.3	49.5	21	35.0	—	—	—
Subgroup 4	70.2	43	24.1	68.6	42	22.2	67.4	35	21.7	65.0	12	24.3
Fatigue‡												
Subgroup 1	47.8	15	22.5	—	—	—	—	—	—	—	—	—
Subgroup 2	42.8	9	18.6	47.4	10	22.8	—	—	—	—	—	—
Subgroup 3	34.1	22	26.7	42.5	21	24.5	47.2	21	27.4	—	—	—
Subgroup 4	39.5	43	24.3	37.9	41	18.3	38.0	35	17.8	42.2	12	24.3
Dyspnea‡												
Subgroup 1	35.3	15	29.3	—	—	—	—	—	—	—	—	—
Subgroup 2	36.7	9	11.0	43.0	10	27.4	—	—	—	—	—	—
Subgroup 3	27.3	23	21.6	25.5	22	20.2	33.1	21	23.5	—	—	—
Subgroup 4	26.1	43	19.8	30.6	42	19.8	34.9	35	19.5	30.3	12	26.2
Insomnia‡												
Subgroup 1	28.7	15	30.4	—	—	—	—	—	—	—	—	—
Subgroup 2	18.3	9	17.4	33.2	10	41.5	—	—	—	—	—	—
Subgroup 3	23.0	23	23.2	27.1	22	26.4	28.3	21	24.1	—	—	—
Subgroup 4	23.1	43	25.6	22.0	42	22.7	19.8	35	21.5	27.5	12	23.7
Social function†												
Subgroup 1	63.2	16	17.5	—	—	—	—	—	—	—	—	—
Subgroup 2	60.8	9	26.5	43.1	10	5.2	—	—	—	—	—	—
Subgroup 3	70.0	23	31.0	73.2	22	27.2	61.6	21	30.3	—	—	—
Subgroup 4	77.2	43	21.0	79.9	42	25.4	76.8	35	23.8	69.1	12	25.6
Overall QoL†												
Subgroup 1	46.3	16	14.9	—	—	—	—	—	—	—	—	—
Subgroup 2	53.6	9	25.8	38.3	10	22.7	—	—	—	—	—	—
Subgroup 3	50.8	23	28.3	54.6	22	28.0	39.3	21	26.6	—	—	—
Subgroup 4	56.9	43	20.2	58.9	41	18.9	56.6	35	20.5	51.3	12	21.5
MHI												
MHI index†												
Subgroup 1	166.5	16	24.4	—	—	—	—	—	—	—	—	—
Subgroup 2	170.7	9	34.7	160.6	10	36.3	—	—	—	—	—	—
Subgroup 3	187.3	23	20.1	185.0	22	25.2	181.0	21	29.6	—	—	—
Subgroup 4	170.2	43	26.0	175.6	42	22.7	177.6	35	22.1	169.4	12	22.8
Psychological distress‡												
Subgroup 1	52.6	16	13.9	—	—	—	—	—	—	—	—	—
Subgroup 2	50.2	9	19.9	55.2	10	22.4	—	—	—	—	—	—
Subgroup 3	39.9	23	9.5	43.1	22	13.3	44.6	21	17.8	—	—	—
Subgroup 4	50.9	43	15.1	46.4	42	12.3	45.3	35	11.5	48.3	12	11.0
Psychological well-being†												
Subgroup 1	53.1	16	11.9	—	—	—	—	—	—	—	—	—
Subgroup 2	54.9	9	16.5	49.8	10	16.3	—	—	—	—	—	—
Subgroup 3	61.2	23	12.6	62.1	22	13.5	59.6	21	14.0	—	—	—
Subgroup 4	54.9	43	12.6	56.0	42	12.5	56.7	35	12.3	51.8	12	12.7
Positive affect†												
Subgroup 1	35.4	16	9.1	—	—	—	—	—	—	—	—	—
Subgroup 2	37.2	9	12.5	33.3	10	13.3	—	—	—	—	—	—
Subgroup 3	41.3	23	10.7	42.4	22	11.2	40.0	21	11.4	—	—	—
Subgroup 4	36.9	43	10.3	37.9	42	9.8	38.0	35	10.0	34.3	12	10.1

NOTE. Subgroups generally coincided with the number of follow-up assessments, as follows: subgroup 1, one assessment or baseline assessment only; subgroup 2, two assessments or baseline + one follow-up at day 50; subgroup 3, three assessments or baseline through second follow-up at day 106; subgroup 4, four assessments or baseline through third follow-up at day 182.

Abbreviation: F/U, follow-up.

*Means for supportive care arm are before cross-over.

†Higher scores indicate better functioning.

‡Higher scores indicate worse symptoms.

Table 3. Means of Selected EORTC QLQ-C30 and MHI Scales for Each Subgroup for Aza C*

	Aza C											
	Baseline			F/U 1			F/U 2			F/U 3		
QoL Scale	Mean	No.	SD	Mean	No.	SD	Mean	No.	SD	Mean	No.	SD
EORTC												
Physical functioning†												
Subgroup 1	53.3	15	22.3	—	—	—	—	—	—	—	—	—
Subgroup 2	68.9	9	20.3	58.9	9	28.5	—	—	—	—	—	—
Subgroup 3	55.8	19	20.6	53.3	18	23.8	66.3	19	28.3	—	—	—
Subgroup 4	64.3	56	28.0	65.0	55	26.7	70.3	54	26.0	77.1	55	4.5
Fatigue‡												
Subgroup 1	47.7	15	21.5	—	—	—	—	—	—	—	—	—
Subgroup 2	37.9	9	18.3	42.8	9	16.0	—	—	—	—	—	—
Subgroup 3	44.1	19	24.5	39.2	18	17.9	38.8	19	19.3	—	—	—
Subgroup 4	39.4	56	24.2	37.0	55	20.0	31.2	54	18.4	26.2	55	21.2
Dyspnea‡												
Subgroup 1	39.6	15	22.3	—	—	—	—	—	—	—	—	—
Subgroup 2	29.3	9	11.0	29.3	9	19.8	—	—	—	—	—	—
Subgroup 3	41.8	19	31.1	42.2	18	22.2	33.0	19	22.0	—	—	—
Subgroup 4	36.0	56	24.6	33.0	55	21.1	28.6	53	23.4	22.0	55	23.0
Insomnia‡												
Subgroup 1	33.0	15	27.9	—	—	—	—	—	—	—	—	—
Subgroup 2	22.1	9	33.3	33.0	9	23.3	—	—	—	—	—	—
Subgroup 3	24.3	19	21.6	22.0	18	25.3	27.8	19	31.8	—	—	—
Subgroup 4	33.7	56	33.2	25.8	55	28.3	17.1	54	22.0	16.2	55	23.7
Social function†												
Subgroup 1	61.8	15	25.6	—	—	—	—	—	—	—	—	—
Subgroup 2	75.7	9	30.3	53.4	9	36.1	—	—	—	—	—	—
Subgroup 3	80.4	19	15.2	68.2	18	26.9	71.6	19	23.7	—	—	—
Subgroup 4	69.6	56	26.6	68.2	55	25.9	71.9	54	22.6	77.3	55	22.4
Overall QoL†												
Subgroup 1	47.1	15	14.4	—	—	—	—	—	—	—	—	—
Subgroup 2	59.1	9	18.8	49.9	9	17.2	—	—	—	—	—	—
Subgroup 3	52.6	19	24.9	54.1	18	19.1	63.3	19	20.1	—	—	—
Subgroup 4	54.0	56	22.9	56.9	55	21.2	65.9	54	18.4	73.3	55	20.1
MHI												
MHI index†												
Subgroup 1	167.6	15	29.2	—	—	—	—	—	—	—	—	—
Subgroup 2	172.2	9	30.9	163.4	9	27.5	—	—	—	—	—	—
Subgroup 3	169.9	18	26.0	172.2	18	25.5	176.9	19	22.0	—	—	—
Subgroup 4	175.3	56	30.7	179.7	55	29.2	184.7	54	25.3	192.7	54	24.8
Psychological distress‡												
Subgroup 1	50.9	15	16.5	—	—	—	—	—	—	—	—	—
Subgroup 2	49.8	9	17.3	52.6	9	14.7	—	—	—	—	—	—
Subgroup 3	52.3	18	15.9	51.0	18	15.2	47.8	19	14.0	—	—	—
Subgroup 4	48.6	56	17.8	45.7	55	17.7	43.2	54	14.4	38.8	54	14.3
Psychological well-being†												
Subgroup 1	52.1	15	14.7	—	—	—	—	—	—	—	—	—
Subgroup 2	56.0	9	15.6	50.0	9	14.1	—	—	—	—	—	—
Subgroup 3	56.2	18	11.9	57.2	18	11.6	58.6	19	9.9	—	—	—
Subgroup 4	57.7	56	14.5	59.4	55	13.3	61.9	54	12.4	65.5	55	11.5
Positive affect†												
Subgroup 1	34.3	15	11.6	—	—	—	—	—	—	—	—	—
Subgroup 2	37.8	9	12.1	33.1	9	11.4	—	—	—	—	—	—
Subgroup 3	37.2	18	10.1	38.4	18	9.8	39.6	19	8.4	—	—	—
Subgroup 4	38.9	56	11.2	40.2	55	10.3	42.3	54	9.8	45.4	55	9.0

NOTE. Subgroups generally coincided with the number of follow-up assessments, as follows: subgroup 1, one assessment or baseline assessment only; subgroup 2, two assessments or baseline + one follow-up at day 50; subgroup 3, three assessments or baseline through second follow-up at day 106; subgroup 4, four assessments or baseline through third follow-up at day 182.

Abbreviation: F/U, follow-up.

*Means for supportive care arm are before cross-over.

†Higher scores indicate better functioning.

‡Higher scores indicate worse symptoms.

Table 4. QoL of Patients Treated With Aza C Versus Supportive Care: Summary of Significant Findings From Linear Regression Analyses

QoL Scale	Supportive Care No.	Supportive Care Slope	Supportive Care SE	Aza C No.	Aza C Slope	Aza C SE	P
EORTC							
Physical functioning*							
Subgroup 1/2	26	−0.12	0.19	24	−0.041	0.19	
Subgroup 3	23	−0.13	0.05†	19	0.11	0.06†	.0002‡
Subgroup 4	43	−0.025	0.02	56	0.07	0.02§	
After cross-over		0.12	0.05				.0040‖
Fatigue¶							
Subgroup 1/2	26	0.11	0.12	24	0.04	0.11	
Subgroup 3	23	0.12	0.05†	19	−0.055	0.05	.0010‡
Subgroup 4	43	0.02	0.02	56	−0.07	0.015§	
After cross-over		−0.14	0.04				.0001‖
Dyspnea¶							
Subgroup 1/2	26	0.18	0.13	24	−0.02	0.01	
Subgroup 3	23	0.05	0.06	19	−0.08	0.06	.0014‡
Subgroup 4	43	0.04	0.03	56	−0.07	0.02§	
After cross-over		−0.16	0.04				.0002‖
Insomnia¶							
Subgroup 1/2	26	0.28	0.20	24	0.14	0.19	
Subgroup 3	23	0.03	0.06	19	0.02	0.065	.035‡
Subgroup 4	43	0.004	0.03	56	−0.089	0.02§	
After cross-over		−0.10	0.04				.025‖
Social functioning*							
Subgroup 1/2	26	−0.38	0.20	24	−0.34	0.20	
Subgroup 3	23	−0.10	0.05	19	−0.07	0.055	.041‡
Subgroup 4	43	−0.04	0.02	56	0.04	0.02†	
After cross-over		0.06	0.04				.156‖
Overall QoL*							
Subgroup 1/2	26	−0.28	0.11#	24	−0.10	0.10	
Subgroup 3	23	−0.11	0.05†	19	0.10	0.05†	.0001‡
Subgroup 4	43	−0.02	0.02	56	0.10	0.02§	
After cross-over		0.15	0.04				.0001‖
MHI							
MHI index*							
Subgroup 1/2	26	−0.20	0.14	24	−0.01	0.13	
Subgroup 3	23	−0.07	0.045	19	0.05	0.05	.025‡
Subgroup 4	43	0.02	0.02	56	0.08	0.015§	
After cross-over		0.08	0.04				.037‖
Psychological distress¶							
Subgroup 1/2	26	0.095	0.08	24	0.04	0.08	
Subgroup 3	23	0.045	0.03	19	−0.04	0.03	.015‡
Subgroup 4	43	−0.02	0.01	56	−0.045	0.01§	
After cross-over		−0.04	0.02				.016‖
Psychological well-being*							
Subgroup 1/2	26	−0.10	0.06	24	−0.10	0.06	
Subgroup 3	23	−0.03	0.02	19	0.01	0.02	.025‡
Subgroup 4	43	0.003	0.01	56	0.04	0.007§	
After cross-over		−0.04	0.02				.016‖
Positive affect*							
Subgroup 1/2	26	−0.07	0.05	24	−0.075	0.05	
Subgroup 3	23	−0.02	0.02	19	0.01	0.02	.0077‡
Subgroup 4	43	0.02	0.008	56	0.03	0.006§	
After cross-over		0.03	0.01				.019‖

*Because higher scores in this measure indicate better functioning, negative slopes indicate worsening status/functioning over time and positive slopes indicate improved status/functioning over time.

†Because higher scores in this measure indicate worse functioning, negative slopes indicate improved status/functioning over time and positive slopes indicate worsening status/functioning over time.

‡Slope for the supportive care and Aza C arms is significantly different from zero at the .05 level of significance.

§Slope for supportive care and Aza C arms is significantly different from zero at the .01 level of significance.

¶Slope for supportive care and Aza C arms is significantly different from zero at the .001 level of significance.

‖The first *P* value in right column represents a test of whether the slopes of the Aza C and supportive care arms are equal before patients crossed over from the supportive care into the Aza C arm.

#Second *P* value in right column represents a test af whether the change in scores after cross-aver is significant.

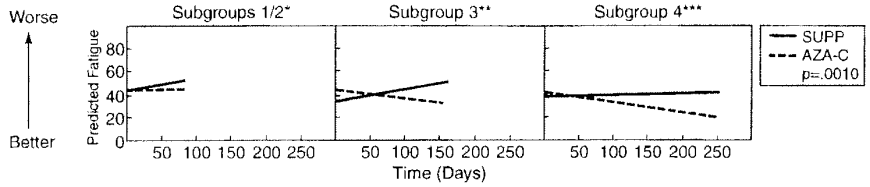

Fig 1. EORTC fatigue subscale by assessment subgroup. *Supportive care (SUPP), n = 26; Aza C, n = 24. **SUPP, n = 23; Aza C, n = 19. ***SUPP, n = 43; Aza C, n = 56. Subgroups generally coincided with the number of assessments.

from baseline was comparable with an improvement in the ECOG score of 0.8 to 0.4. For supportive care patients in subgroup 3, the comparable ECOG performance status ratings in relation to the EORTC physical functioning scores worsened from 1.1 to 1.5 ECOG ratings, whereas those in subgroup 4 were minimally worse, going from 0.4 to 0.6 (higher EORTC scores indicate an improvement in functioning; lower ECOG scores indicate an improvement in performance status) (Table 5).

In addition, the mean change in the EORTC physical functioning subscale for Aza C subgroup 3 was 10.5, whereas patients in the supportive care arm had a mean decrease of −11.5 (Table 5). With a 10-point change on the EORTC having been determined to be comparable to a patient's rating of a little improvement,[14,15] on average, there was a clinically significant improvement in physical functioning for patients in subgroup 3 who had been treated with the full four cycles of Aza C.

In a related set of analyses, the percentage of patients in subgroups 2, 3, and 4 whose EORTC score improved by 10 points or greater at follow-up from baseline was calculated for those subscales found to be significantly different by treatment arm over time in the above analyses (Table 6). For subgroups 3 and 4, by day 106, the percentage of patients treated by Aza C whose EORTC score improved by 10 points or more from baseline was larger than the percentage of patients receiving supportive care, often by differences of 20% or greater. By day 182, this trend continued for all measures except fatigue, with an even larger percentage of Aza C patients having an improved EORTC score of 10 points or more than those receiving supportive care. The percentage improvement in subgroup 2 in EORTC symptom scores of 10 points or more at day 50 was greater in those treated by supportive care than Aza C, in the opposite direction than what was hypothesized. However, these differences were not statistically significant.

Based on MHI psychological distress scores, patients in subgroups 2 through 4 were classified into low to moderate distress (< 1.5 SD below the population norm) versus high distress (≥ 1.5 SD above the population norm). At baseline, 16% (13 of 84) of the Aza C patients were in high distress, whereas at day 106, 6% (five of 84) patients were in high distress. There was no change in the percentage of supportive care patients in high distress over this time, with 8% (six of 76) in high distress at both baseline and day 106.

Last, the quality-of-life measures were examined in relation to treatment response for patients in subgroups 3 and 4 combined (n = 66) in only the Aza C arm, because there were virtually no responders in the supportive care arm.[5] A linear random coefficient model was used, with treatment response entered as a covariate. No significant relationships were found between treatment response and any of the quality-of-life measures.

Placebo and Hawthorne Effects

Because the research design involved a nonblinded randomization to Aza C or supportive care, it was possible that the significant differences found in quality

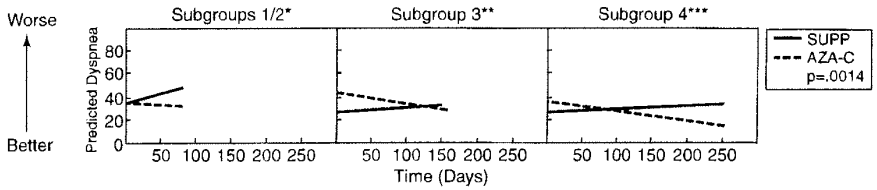

Fig 2. EORTC dyspnea subscale by assessment subgroup. *Supportive care (SUPP), n = 26; Aza C, n = 24. **SUPP, n = 23; Aza C, n = 19. ***SUPP, n = 43; Aza C, n = 56.

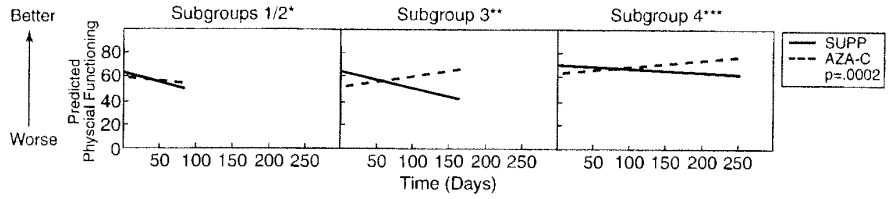

Fig 3. EORTC physical functioning subscale by assessment subgroup. *Supportive care (SUPP), n = 26; Aza C, n = 24. **SUPP, n = 23; Aza C, n = 19. ***SUPP, n = 43; Azo C, n = 56.

of life between treatment arms could be attributed to either a placebo effect,[17] that is, a psychological improvement attributable to receiving any active treatment with the expectation that it would be effective, or the Hawthorne effect,[18] an improvement in functioning attributable to the extra attention paid by the medical staff to patients receiving Aza C. If the findings had been largely attributable to placebo or Hawthorne effects, quality-of-life ratings of nonresponders in the Aza C arm would have improved significantly by the second assessment at day 50. Therefore, the four quality-of-life measures responsible for most of the significant findings were examined in Aza C nonresponders in subgroups 3 and 4 (n = 19) to test for the placebo and Hawthorne effects. At the second assessment, the MHI psychological distress mean scores showed negligible changes from baseline levels, the EORTC physical functioning scores showed a worsening from baseline levels by approximately 0.8 SD (in the opposite direction of placebo and Hawthorne effects), and the mean scores of the EORTC dyspnea and fatigue subscales showed some minor but nonsignificant improvement from baseline levels by approximately 0.10 to 0.15 SD.

DISCUSSION

Patients' quality of life improved significantly with Aza C treatment compared with supportive care, as evidenced by decreased fatigue and dyspnea and improved physical functioning and positive affect. These very same areas of functioning showed a marked improvement in the patients who began on supportive care and crossed over to treatment. The significant interrelationship between physical status and psychological state at baseline illuminated the likely reason for the constellation of significant effects related to Aza C: as physical status improved with Aza C, so did patients' psychological state. These improvements in quality of life paralleled the clinical findings of the trial, with patients treated with Aza C having a significantly better treatment response (complete response, partial response, or improved: Aza C, 60%; supportive care, 5%; $P < .001$) and reduced frequency of and transformation to AML or death (Aza C, 15%; supportive care, 38%; $P = .001$) than those on supportive care.[5] Underscoring the central role of treatment response in driving quality of life, patients experiencing the greatest improvements in their quality of life were those in subgroups 3 and 4 who received four cycles of Aza C, the number of cycles that had been determined to obtain the maximum effects of Aza C in the phase II trial.[2]

In a series of post hoc analyses, significant differences in physical functioning, physical symptoms, and psychological state were associated with clinically significant improvements for patients treated with Aza C. Using EORTC physical functioning ratings in relation to ECOG performance status ratings, by day 182, patients in subgroup 3 treated with Aza C could expect to have their physical functioning improve over the course of treatment from an estimated ECOG rating of 1.3 to 0.7, an ECOG level that is greater than symptomatic but nearly

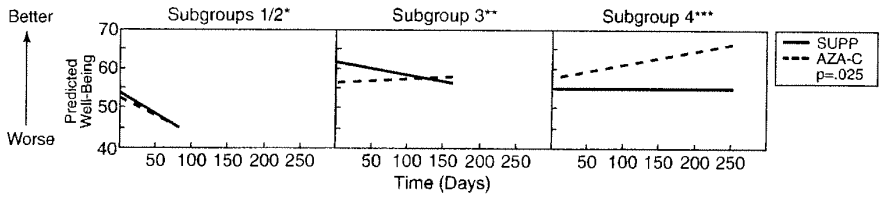

Fig 4. MHI physiological well-being subscale by assessment subgroup. *Supportive care (SUPP), n = 26; Aza C, n = 24. **SUPP, n = 23; Aza C, n = 19. ***SUPP, n = 43; Aza C, n = 56.

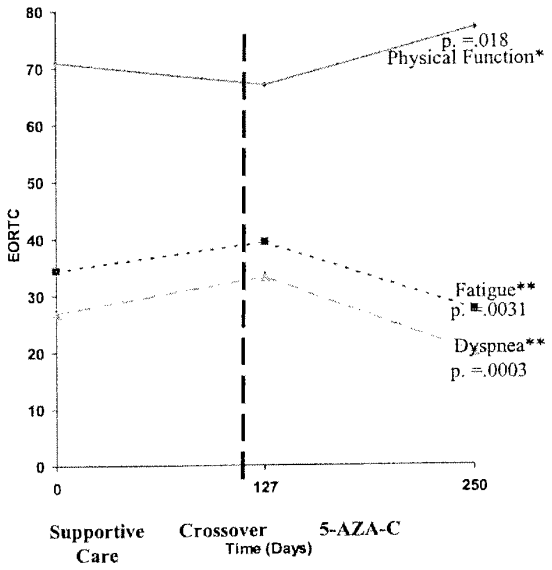

Fig 5. EORTC fatigue, dyspnea, and physical functioning of patients who cross over from supportive care to Aza C (n = 30). *Higher scores indicate better functioning. **Lower scores indicate symptom improvement.

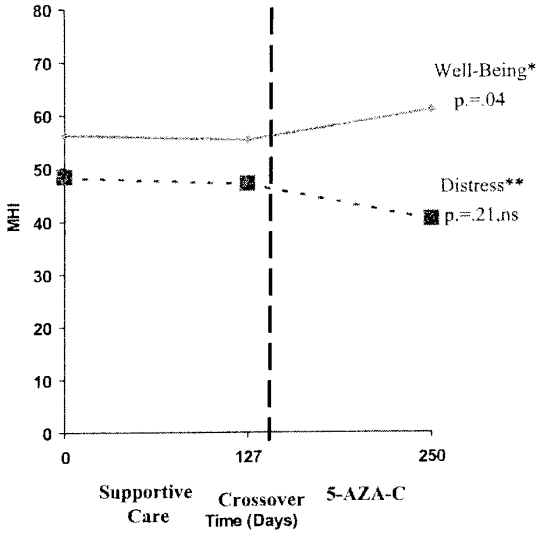

Fig 6. MHI physiological distress and well-being of patients who cross over from supportive care to Aza C (n = 30). *Higher scores indicate better well-being. **Lower scores indicate less distress.

fully ambulatory. A patient on supportive care in subgroup 3 could expect his or her physical functioning to worsen over the course of treatment, from an estimated ECOG rating of 1.1 to 1.5, going from symptomatic but nearly fully ambulatory to halfway between ECOG ratings 1 and 2, with a rating of 2 defined as spending some time in bed but less than 50% of time.

Furthermore, 20% or more of Aza C than supportive care patients in subgroups 3 and 4 had an improved EORTC score by 10 points or more from baseline to follow-up (the magnitude of change that had been determined to be a clinically significant improvement)[14,15] in physical functioning, three physical symptom subscales, and overall quality of life. Last, based on the MHI data, a patient in the Aza C group would have a 10% greater likelihood that by the end of treatment his or her psychological distress would be reduced to a level not requiring additional evaluation and treatment. For the average patient in the supportive care group, no such improvement could be expected.

In an open-label randomized trial in which one of the arms is supportive care, there is a concern that the statistical findings may have been attributable to either placebo or Hawthorne effects. Although a double-blinded design would have controlled for these effects, it was not used because of ethical concerns. Patients in the supportive care group would have been required to have subcutaneous saline injections to blind the treatment conditions, exposing neutropenic and thrombocytopenic patients to possible infections or bleeding.

Table 5. Clinical Significance of EORTC Physical Functioning Scores

EORTC Physical Functioning*	Baseline (SD)	Follow-Up at Day 106 (SD)	Change (SD)	Comparable Change in ECOG†
Subgroup 3				
Aza C (n = 19)	55.8 (20.6)	66.3 (28.3)	10.5 (27.8)	1.3-.7
Supportive care (n = 21)	61.0 (28.6)	49.5 (35.0)	−11.5 (24.1)	1.1-1.5
Subgroup 4				
Aza C (n = 54)	64.4 (27.4)	70.3 (26.0)	5.9 (26.8)	0.8-0.4
Supportive care (n = 35)	70.3 (24.9)	67.4 (21.7)	−2.9 (22.3)	0.4-0.6

*Higher ratings in the EORTC functioning subscale indicate an improvement in physical functioning.

†ECOG performance status scores at baseline of 0 (normal activity), 1 (symptomatic but nearly fully ambulatory), and 2 (some time spent in bed but less than 50% of the day) were associated with EORTC physical functioning subscale mean ratings of 74.9, 62.7, and 38.33, respectively. Higher ratings in ECOG scores indicate a worsening performance status.

Table 6. Improvement in EORTC Subscale Scores From Baseline by ≥ 10 Points by Treatment Arm for Subgroups 2, 3, and 4

		Change From Baseline to Follow-Up			
		Supportive Care		Aza C	
Subgroup/EORTC Subscale	Day	%	n/N	%	n/N
Subgroup 2					
Physical functioning	50	0	0/10	22.2	2/9
Dyspnea	50	20	2/10	11.1	1/9
Fatigue	50	30	3/10	11.1	1/9
Insomnia	50	30	3/10	11.1	1/9
Overall QoL	50	0	0/10	0	0/9
Subgroup 3					
Physical functioning	50	18.2	4/22	16.7	3/18
	106	23.8	5/21	47.4	9/19
Dyspnea	50	13.6	3/22	11.1	2/18
	106	9.5	2/21	42.1	8/19
Fatigue	50	9.0	4/21	33.3	6/18
	106	19.0	4/21	47.4	9/19
Insomnia	50	13.6	3/22	27.8	5/18
	106	9.5	2/21	36.8	7/19
Overall QoL	50	27.3	6/22	22.2	4/18
	106	14.3	3/21	47.4	9/19
Subgroup 4					
Physical functioning	50	19.0	8/42	20.0	11/55
	106	20.0	7/35	37.0	20/54
	182	25.0	3/12	45.5	25/55
Dyspnea	50	4.8	2/42	25.5	14/55
	106	2.9	1/35	35.8	19/53
	182	16.7	2/12	47.3	26/55
Fatigue	50	41.5	17/41	38.2	21/55
	106	28.6	10/35	51.9	28/54
	182	58.3	7/12	63.6	35/55
Insomnia	50	16.7	7/42	32.7	18/55
	106	11.4	4/35	44.4	24/54
	182	8.3	1/12	38.2	21/55
Overall QoL	50	22.0	9/41	29.1	16/55
	106	22.9	8/35	44.4	24/54
	182	33.3	4/12	58.2	32/55

Post hoc testing of placebo and Hawthorne effects on nonresponders in the Aza C group demonstrated no significant improvement in nonresponders' quality of life from baseline to day 50. Thus, it is unlikely that either the placebo or Hawthorne effects were of sufficient magnitude to account for the marked differences in patients' physical symptoms and quality of life found between treatment arms.

Several factors argued for a valid treatment effect in patients' quality of life. First, there were multiple quality-of-life parameters in which differences were found to be significant, many of which were highly significant ($P = .008$ to $P < .0001$). Second, significant differences between treatment arms were not found on all measures (eg, EORTC pain, insomnia, diarrhea, and cognitive functioning), as would have been the case if there had been a blanket placebo effect. Third, the most significant differences between arms were found in patients who remained on treatment for four cycles of Aza C, commensurate with the number of cycles found to be related to treatment response.[2] Last, the significant differences in quality of life were consistent with the clinical findings of the trial.[5]

Because the clinical significance of quality-of-life ratings from the different measures was an important aspect of this research, a series of post hoc analyses were generated using a minimum change of 10 points or greater in the EORTC scale as an indicator of a clinically significant improvement and benchmark values of the EORTC physical functioning scale for the different ECOG performance levels obtained at

baseline. These benchmark values would need to be replicated in future studies to validate them as indicative of clinical improvement in physical functioning for the populations of patients with MDS as well as other populations of patients with cancer.

Last, because a substantial proportion of supportive care patients had crossed over to the Aza C arm before completing all four assessments, with only 13% (n = 12) remaining in the supportive care arm by day 182, we were restricted to using linear regressions, where a substantial number of patients on each arm had the first two assessments. It is possible that with a larger number and percentage of patients remaining in the supportive care arm, with all four quality-of-life assessments completed, other trajectories of change over time might well have emerged.

In conclusion, these results demonstrated that the quality of patients' lives was significantly improved by their treatment with Aza C. Not only was there a significant improvement in patients' fatigue, dyspnea, physical functioning, and psychological state, but also inroads were made concerning the translation of these findings into more clinically meaningful and understandable terms. Furthermore, with the quality-of-life results paralleling patients' significantly greater improvement for those on Aza C versus supportive care in terms of hematologic response, decreased frequency, and delayed time to transformation to AML or death,[5] the meaning of a clinical response has also been more powerfully conveyed in relation to patients' functioning. Clinical and quality-of-life data have made it evident that Aza C is an important treatment option for MDS.

APPENDIX

The appendix listing participating institutions and investigators is available online at www.jco.org.

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Phase Specificity of 5-Azacytidine against Mammalian Cells in Tissue Culture[1]

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SUMMARY

5-Azacytidine (1 to 5 μg/ml) significantly inhibited mitosis of L1210 cells in culture only after 2 hr of incubation. The inhibition of mitosis was correlated to the inhibition of DNA synthesis.

With asynchronous DON and L1210 cells, the cell-kill (inhibition of proliferative capacity) reached a saturation value at high doses of the drug. Any further increase in drug concentration did not result in a corresponding increase in percentage of cell-kill. This indicated that 5-azacytidine kills cells in certain phases of the cell cycle. With synchronous DON cells, it was shown that the drug was lethal predominantly in the S phase.

5-Azacytidine caused considerable chromosome damage when L1210 cells in culture were exposed to the drug at 5 μg/ml for 2 hr at 37°.

INTRODUCTION

Some antitumor agents have selective biological and/or biochemical effects (1, 8, 9, 11–13, 16, 21) at certain phases of the cell cycle. Cytosar [cytarabine, (The Upjohn Co., Kalamazoo, Mich.) cytosine arabinoside, ara-C] and hydroxyurea inhibit cells primarily in the S phase, while vinblastine is effective only in the mitotic stage. Several agents, such as γ-radiation and nitrogen mustard, lack this phase specificity and kill cells in all phases of the cell cycle. Such information, for instance, was used by Skipper *et al.* (18) in designing effective dosage schedules for ara-C.

Since 5-azaCR[2] inhibited DNA synthesis more than RNA synthesis (10), the action of the drug on asynchronous L1210 cells and asynchronous and synchronous DON cells was studied to determine whether the drug specifically inhibited a certain phase of the cell cycle. The effect of the drug on the chromosomal integrity and the inhibition of mitosis of L1210 cells is also reported.

MATERIALS AND METHODS

Tissue Culture Systems. L1210 cells were maintained in culture in RPMI 1634 medium (Grand Island Biological Company, Grand Island, N. Y.),[3] developed by Moore *et al.* (14). The cells were used at 5×10^5 cells/ml (in logarithmic phase of growth) in most cases. The incorporation of radioactive precursors, *e.g.*, TdR-^{3}H and uridine-^{3}H, into respective macromolecules of intact L1210 cells was determined by the method described elsewhere (10).

DON cells, a Chinese hamster fibroblast line (American Type Culture Collection, No. CCL 16), were grown in McCoy's 5A medium modified by the addition of lactalbumin hydrolysate (0.8 g/liter) and fetal calf serum (200 ml/liter) (Grand Island Biological Co.). The cell monolayer was detached from glass by treatment with a 0.1% trypsin solution. The cells were dispersed and grown in 8-oz. bottles planted with about 2×10^6 cells in 25 ml medium. The cells were maintained in the logarithmic growth phase by subculturing every 2 days.

Chromosome Preparation. Chromosome preparations were made by the air-drying method described by Tijo and Whang (20). After exposure to 5-azaCR for a given period, the cells were centrifuged at 500 rpm for 2 min in a clinical centrifuge. The loosely packed cells were then suspended in 5 ml of hypotonic solution (1% sodium-citrate, pH 7) and allowed to stand for 10 min. The cells were centrifuged, and the loose pellet was resuspended in fixative (ethanol:glacial acetic acid, 3:1). After being fixed for 20 min, the cells were centrifuged, and the pellet was resuspended in a small amount of fresh fixative. A couple of drops of the cell suspension were placed on a clean glass slide and dried by an air stream. The cells were stained by an aceto-orcein stain (6). Two slides were prepared per sample for determination of chromosome damage and MI. Approximately 1000 cells per slide were counted to obtain the MI.

Determination of Cell Cycle of L1210 and DON Cells in Tissue Culture. Mean transit times through the cell cycle of both cell lines were determined from plots of labeled mitoses at various times after pulse labeling with TdR-^{3}H (15).

[1]This investigation was supported in part by Contract PH43-68-1023 with Chemotherapy, National Cancer Institute, NIH, Bethesda, Md.

[2]The abbreviations used are: 5-azaCR, 5-azacytidine; TdR, thymidine; MI, mitotic index.

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[3]RPMI medium is medium developed at Roswell Park Memorial Institute, Buffalo, N. Y., for growing human and mouse leukemic cells.

Determination of Percentage of Survivors after Exposure of L1210 Cells to 5-AzaCR. L1210 cells were exposed to 5-azaCR in a series of concentrations for varying periods. The cells were centrifuged and resuspended in fresh medium, and 10^6 cells were injected i.p. into female BDF_1 mice (10 to 20 mice/group). The control (not drug-treated) cells were injected into mice at concentrations ranging from 10^3 to 10^6 cells. The median day of death was determined according to protocols established by Chemotherapy, National Cancer Institute (4). The percentage of cell-kill was determined by comparing the life-span (median day of death) of animals given injections of 5-azaCR-treated cells to a standard curve showing the relationship between injected untreated cells and the life-span of the corresponding animals. The BDF_1 [(C57BL/6 ♀ × DBA/2 ♂)F_1] mice (20 g ±2) were obtained from Jackson Memorial Laboratory, Bar Harbor, Maine.

The percentage of survival of drug-exposed cells was also determined by the cloning methods of Himmelfarb *et al.* (7).

Determination of Phase Specificity of 5-AzaCR in Both Asynchronous and Synchronous DON Cells. A synchronous culture of DON cells was prepared from mitotic cells harvested by the procedure described by Stubblefield *et al.* (19). Cells were grown in 32-oz. prescription bottles and mitotic cells were accumulated by exposure to 0.06 μg/ml Colcemid for 3 hr. Mitotic cells were harvested by shaking the bottles for 1 min (reciprocating shaker, 88 strokes/min, 2 inch/stroke) with 40 ml of cold 0.125 mg/ml trypsin, and the mitotic cells were accumulated by centrifugation. About 10^6 cells were planted in 3-oz. bottles (Duraglas, Owens-Illinois Co., Toledo, Ohio) in 10 ml of fresh medium and briefly gassed with a 5% CO_2-95% air mixture. The cells were then exposed to 5-azaCR for 2 hr at different times after planting to expose cells in different parts of the cell cycle.

For experiments with asynchronous cells, 10^6 cells in logarithmic growth were planted in 3-oz. prescription bottles. After a 24-hr incubation, the monolayer cultures were exposed to different concentrations of 5-azaCR for 3 or 5 hr. After exposure to 5-azaCR, the medium was poured off, and the cells were detached with trypsin, centrifuged, and resuspended in fresh medium. The cells were diluted in warm medium, and about 40 cells were plated in plastic Petri plates (Linbro Chemical Co., New Haven, Conn.) and incubated in an atmosphere of 8% CO_2-92% air for 7 days (37°). Then the medium was removed by suction, and the colonies were stained with 0.2% methylene blue in 70% ethanol and counted with a Quebec Colony counter (Spencer Lens Co., Buffalo, N. Y.). The plating efficiencies were about 40 to 50% for synchronous cells and 60 to 80% for asynchronous cells.

RESULTS

Effect of 5-AzaCR on Macromolecule Synthesis. The effect of 5-azaCR on polynucleotide synthesis in intact L1210 cells is shown in Chart 1 and indicates that DNA synthesis was inhibited to a greater extent than RNA synthesis at all doses used. Similar patterns of inhibition were also seen with DON cells (Chart 2).

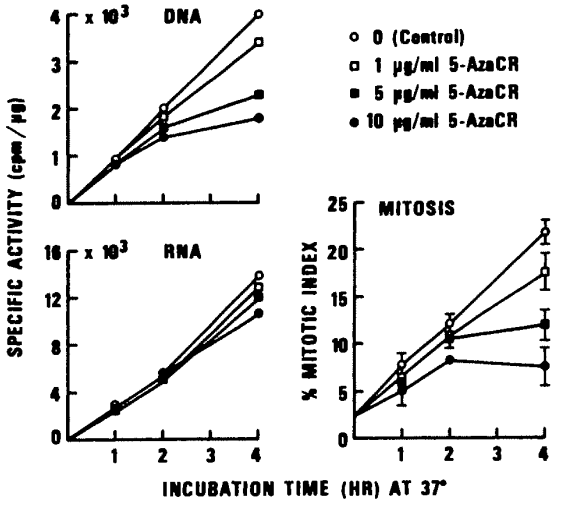

Chart 1. Inhibition of polynucleotide synthesis and mitosis of L1210 cells in culture by 5-azaCR. L1210 cells (*circa* 5 × 10^5/ml) were incubated with 5-azaCR and labeled metabolite (TdR-^{3}H or UR-^{3}H, 0.64 μCi/2.8 μg/ml cells) for 1 to 4 hr. The acid-insoluble polynucleotide fractions were assayed for cpm and nucleic acid content. L1210 cells in culture were exposed to different concentrations of 5-azaCR and Colcemid (0.16 μg/ml). Samples were taken at 1, 2, and 4 hr, and 2 slides were prepared per sample. Approximately 1000 cells were counted per sample.

Inhibition of Mitosis of L1210 Cells by 5-AzaCR. In order to accurately determine the MI, Colcemid was added to cells in logarithmic growth to accumulate cells in metaphase. Four experiments were done, and, since the results appeared to be quite reproducible, a representative example is shown in Chart 1. At 1 μg/ml of 5-azaCR, MI was not inhibited during the first 2 hr of incubation, and 30 to 50% inhibition was observed during the next 2 hr. Even at 5 μg/ml, MI was not significantly affected for the first 2 hr, but the passage of cells to mitosis was almost completely blocked after 2 hr of exposure to the drug. The inhibition of DNA synthesis, but not of RNA synthesis, parallels the inhibition of mitosis of L1210 cells, as seen in Chart 1.

Effect of 5-AzaCR on Proliferation Capacity of Asynchronous L1210 Cells. Chart 3 shows that the percentage of cell survival reaches a constant saturation value for each period of exposure. Cell survival was determined by injecting drug-treated and untreated cells into mice and comparing the respective median days of death.

Similar results were obtained when the number of cells surviving a 1-hr exposure to drug was determined by cloning, as seen in Chart 3.

Effect of 5-AzaCR on Proliferation Capacity of Asynchronous and Synchronous DON Cells. Chart 4 shows that when asynchronous DON cells were exposed to 5-azaCR the percentage of cell-kill reached a constant saturation value for each period of exposure, indicating that 5-azaCR kills cells in certain phases of the cell cycle. This possibility was con-

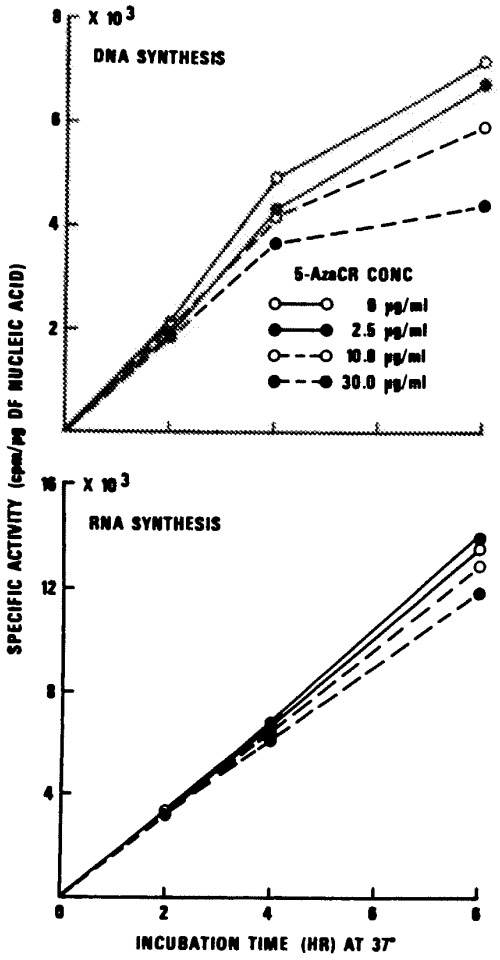

Chart 2. Inhibition of polynucleotide synthesis of DON cells in culture by 5-azaCR. Cells (*circa* 2 X 10^6 cells/8-oz. prescription bottle) were incubated with 5-azaCR and labeled metabolite (TdR-^{3}H or UR-^{3}H, 6.4 μCi/4.6 μg/ml of reaction system) at 37° for 2 to 8 hr. After incubation, the radioactive medium was poured off, and cells (attached to the glass) were washed with fresh medium containing a high concentration (10 mg/ml) of either nonlabeled TdR or uridine. After a quick rinse with 2 ml of trypsin (1 mg/ml), cells were detached from the glass by incubation with 0.5 ml of trypsin (1 mg/ml) for *circa* 5 min and then suspended in 10 ml of fresh medium. Acid-insoluble fractions were obtained by extracting the washed cell pellet twice with 1 ml of 0.5 N perchloric acid (70°C) and used for the determination of radioactivity and nucleic acid content.

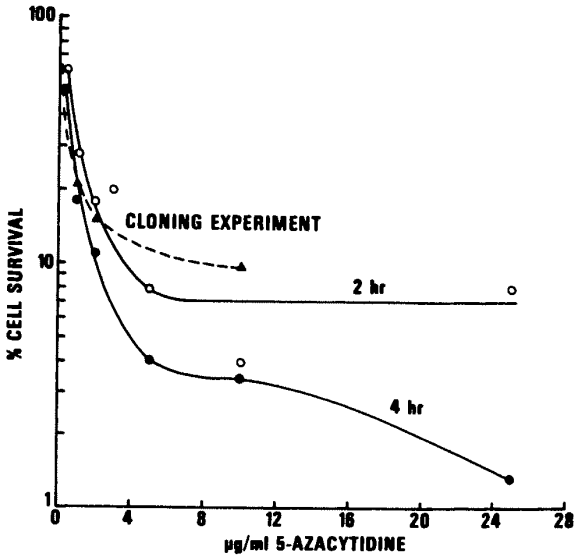

Chart 3. Survival of asynchronous L1210 cells after exposure to 5-azaCR. —, *in vivo* (animal) experiment; - - -, *in vitro* experiment. For animal studies, cells were exposed to 5-azaCR for 2 or 4 hr. The cells were centrifuged and resuspended in fresh medium to give 10^6 cells/ml, and 1 ml of cells were injected into mice (10 to 20 mice/group). The number of viable leukemic cells was determined by comparing the median days of death of mice injected with drug-treated cells to those injected with control (no drug) cells. Data presented in this chart are collective results of 4 experiments differing only in 5-azaCR concentration. For cloning experiments, to determine cell survival by the cloning technique, the following protocol was used. Cell suspensions were centrifuged after 1 hr of exposure to the drug, and the cells were resuspended in medium containing 5% calf serum to give a cell concentration of 10^5/ml. Cells were further diluted in medium containing 20% serum. The cells were finally planted at 5 ml for each 10 X 1.50-cm tube in medium containing 20% calf serum and 0.2% agar. After 8 to 10 days incubation at 37° in a 5% CO_2 atmosphere, the colonies were visually counted. The plating efficiency was about 50%. The % coefficient of variation was about 25 to 30% of the mean value in the cloning experiment.

firmed when synchronously growing DON cells were exposed to 5-azaCR (Chart 5). The results clearly indicate that the percentage of cell-kill is minimal during M and G_1 phases and increases as the cells progress into S phase.

Chromosome Aberrations of L1210 Cells on Exposure to 5-AzaCR. Since 5-azaCR markedly inhibited DNA synthesis, the effect of the drug on L1210 cell chromosomes was studied. The results show that at the 5 μg/ml level and up to 3 hr (37°) this agent causes sticky chromosomes, achromatic gaps, and chromosome fragmentation of L1210 cells in culture (Fig. 1).

DISCUSSION

Fučik *et al.* (5) reported that 5-azaCR caused chromosomal mutation in the root meristem of *Vicia faba* and assumed the mutagenic activity of this compound was associated with its incorporation into DNA. The drug inhibited both DNA and RNA synthesis, was incorporated into the polynucleotides in L1210 cells (10), and caused sticky chromosomes, achromatic gaps, and chromosome fragmentation in these cells (Fig. 1).

Our results (Chart 1) showed that the inhibition of DNA synthesis (but not of RNA synthesis) parallels the inhibition of mitosis of L1210 cells; RNA synthesis is much less

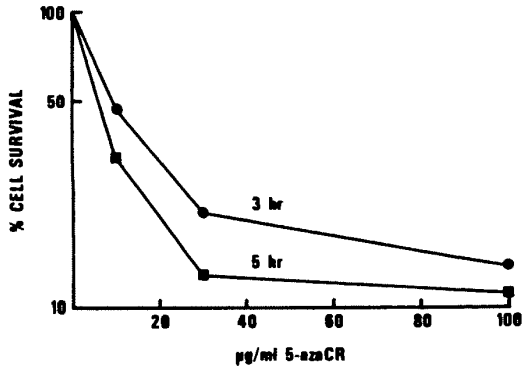

Chart 4. Survival of asynchronous DON cells after exposure to 5-azaCR. Cell monolayers were exposed to varying concentration of the drug for 3 to 5 hr. The drug was removed, and the cells from each sample were diluted and planted in 12 Petri plates. After 7 to 8 days incubation, the colonies were stained and counted. The percentage of cell survival was determined by comparing the percentage of surviving colonies in drug-treated samples to those in control samples. The cloning efficiency in the control (no drug) samples was about 78%; this represented 100% survival. The % coefficient of variation was about 20% of the mean value.

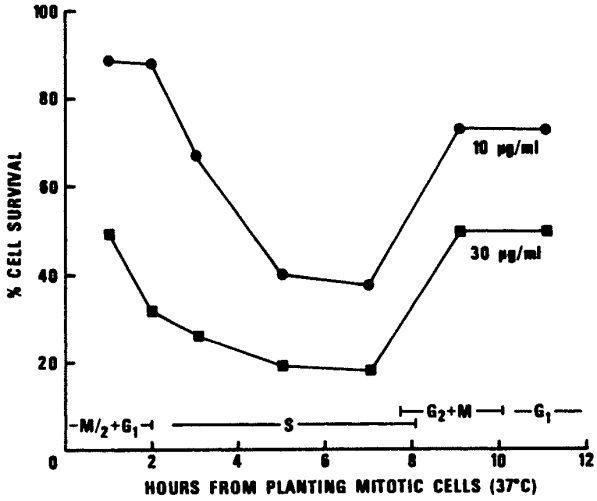

Chart 5. Survival of synchronous DON cells after exposure to drug. Mitotic cells were harvested from monolayers of DON cells, after pretreatment with Colcemid for 3 hr, and were used to start a synchronous culture. The cells were exposed to drug at different times after planting for 2 hr, and then the percentage of surviving cells was determined, as in Chart 4. The % coefficient of variation was about 20% of the mean value.

inhibited by this agent. Since the length of G_1, S, G_2, and M phases of L1210 cells in culture are, respectively, 1.2, 8.2, 1.2, and 0.6 hr (B. K. Bhuyan, unpublished data), these results indicated that cells previously in late S phase (2 hr prior to M phase) are blocked from proceeding to mitosis.

Bruce *et al.* (2, 3) have shown that with phase-specific agents, once the inhibitory concentration is reached, the percentage of cell-kill reached a constant saturation value for that period of exposure. With a longer period of exposure, more cells enter the sensitive phase and are killed, resulting in a higher saturation value for the percentage of cell-kill. Such data were obtained with presumably phase-specific agents such as Cytosar by Karon and Shirakawa (9) and with vinblastine, TdR-^{3}H, and hydroxyurea by Bruce *et al.* (2, 3). With phase-nonspecific agents, however, an exponential cell-kill pattern is seen, and a saturation value for the percentage of cell-kill will not be obtained, as observed by Bruce *et al.* (2) with γ-radiation and nitrogen mustard.

Chart 3 illustrates the relationship between 5-azaCR dose and percentage of cell survival of asynchronous L1210 and DON cells, respectively. A saturation value of the percentage of cell-kill was obtained in both cell lines studied. In L1210 cells, a plateau of cell-kill was seen in both cloning and animal experiments when 5 μg/ml or higher concentrations of 5-azaCR were used. It must be realized, however, that results from the animal experiments are based on the assumptions that the drug-induced increase in host life-span results from the death of a high percentage of the L1210 cells and not from an increase in either the generation time or the lag phase of drug-treated L1210 cells. These possibilities have been appropriately discussed by Skipper *et al.* (17). In DON cells, the percentage of cell-kill becomes constant (about 90%) when the cells are incubated with 30 μg/ml 5-azaCR or more for 3 to 5 hr (37°). These results are in agreement with the previous findings, which suggest 5-azaCR acts more or less as a phase-specific agent, killing cells predominantly in a certain phase of the cell cycles of both cell lines studied.

The percentage of cell-kill of a synchronous DON cell population is minimal during M and G_1 phases and increases as the cells progress into S phase. In the synchronous culture, there was a burst of mitosis about 8 hr after the mitotic cells were planted. When the synchronous culture was pulse-labeled with TdR-^{3}H the percentage of labeled cells was 0, 72, 85, 88, 90, 62, and 44% at 1, 2, 3, 4, 6, 8, and 10 hr, respectively, after planting mitotic cells. This indicates that, while synchrony was maintained during G_1 and early S phases, the cells in G_2 phase are contaminated with a large percentage of S phase cells. Therefore, although it seems that percentage of cell survival decreases during G_2 phase, it cannot be so stated. It is fairly obvious that 5-azaCR acts as an S phase-specific agent against DON cells, presumably through its greater inhibition of DNA synthesis (Chart 2). The shape of these curves (Chart 5) is similar to those obtained for Cytosar, a known S phase-specific agent (B. K. Bhuyan, unpublished data).

Work is in progress to determine the cell-cycle specificity of 5-azaCR upon a synchronous culture of L1210 cells. However, by carefully comparing the results of L1210 cells (Charts 1 and 3) with those obtained from DON cells (Charts 2, 4, and 5), it is reasonable to assume that 5-azaCR acts principally as an S phase-specific agent against leukemic L1210 cells in culture. Although this antimetabolite inhibits both DNA and RNA synthesis in leukemia L1210 systems, presumably via its incorporation into the respective molecules (10), the marked effect on DNA molecules

appears to be one of the primary determinants of cytotoxicity.

ACKNOWLEDGMENTS

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Fig. 1. Chromosome aberrations in 5-azaCR-treated cells. L1210 cells were exposed to 5 μg/ml 5-azaCR for 2 to 3 hr. The cells were centrifuged and resuspended in warm medium containing Colcemid for 1 hr, and then slides were prepared. (*A*) Control (no drug) cells; (*B*) note achromatic gaps; (*C*) and (*D*) sticky and elongated chromosomes; and (*E*) fragmented chromosomes.

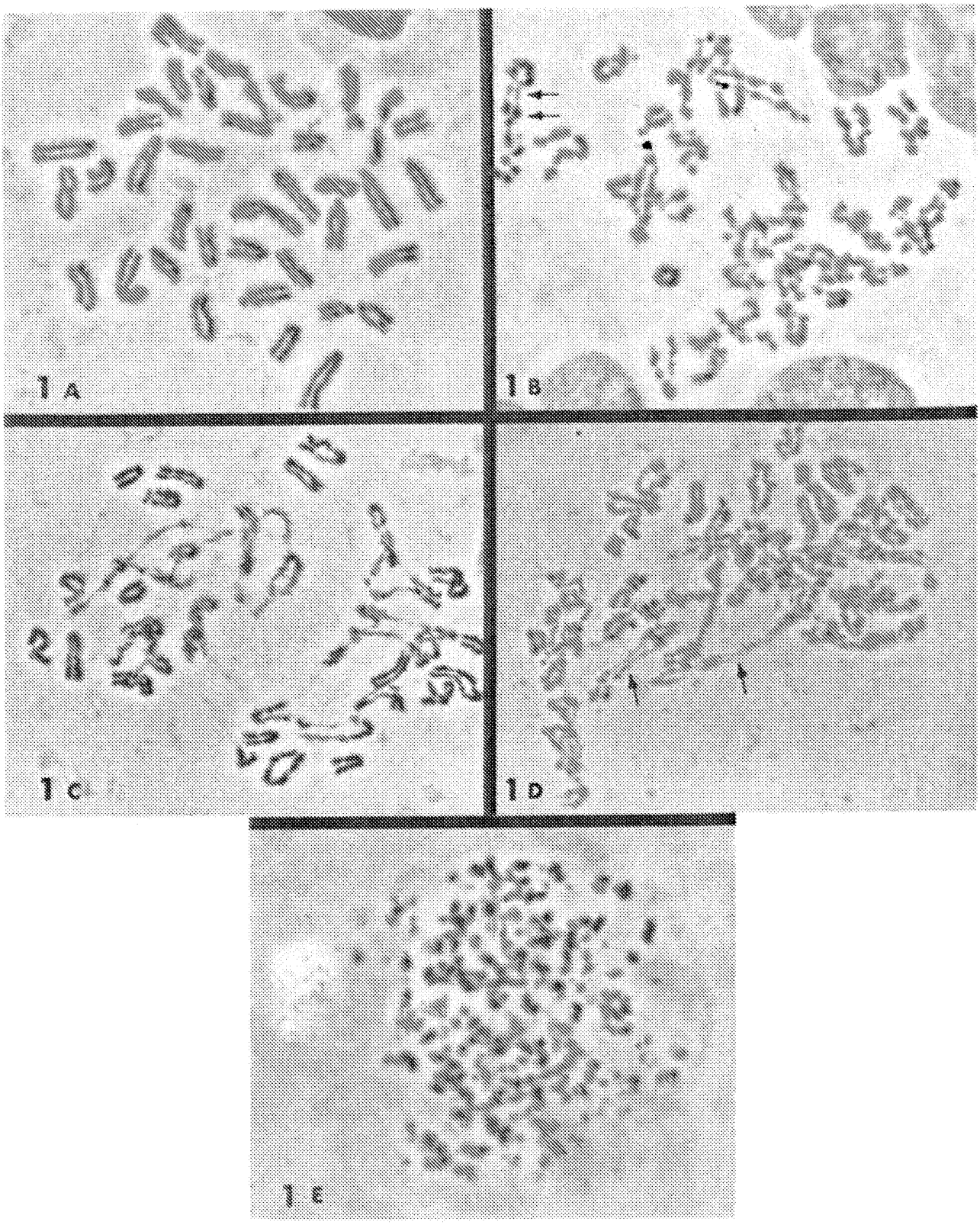

Table IX—Extent of Complex Formation between 3,4-Dimethylphenol and Ethyl Myristate Determined by Partitioning Study

Total Concentration of 3,4-Dimethylphenol, $\times 10^2 M$	Partition Coefficient	$\frac{[DMP_{comp}]}{[DMP_0]}$
0	1.37	—
1.53	1.73	0.27
3.07	1.96	0.43
6.14	2.61	0.91
9.21	3.31	1.43
15.03	4.57	2.35
30.00	7.94	4.83

for various cosolvent systems, and each showed a straight-line relationship between the two parameters (Fig. 4). The stability constants, $K_{1:1}$ and $K_{1:2}$, for each ester were calculated from Fig. 4 and are shown in Table VII. It is evident from these results that 4-hexylresorcinol forms not only 1:1 but also 1:2 complexes with esters in hexane. The stability constant values obtained for ethyl myristate were somewhat higher than those for the other esters. This result is probably due to the fact that ethyl myristate has a larger hydrocarbon chain, which results in a better interaction with the hydrophobic portion of phenols.

To ascertain whether the formation of 1:2 complexes is due to the involvement of the two hydroxy groups of 4-hexylresorcinol, the partitioning study was repeated with 3,4-dimethylphenol. The data obtained from this study are shown in Tables VIII–X. A plot of $[DMP_{comp}]/[DMP_0][E_T]$ *versus* the total ester concentration is shown in Fig. 5; $[DMP_{comp}]$ is the concentration of 3,4-dimethylphenol in the complex form, and $[DMP_0]$ is the concentration of the free form.

Table X—Extent of Complex Formation between 3,4-Dimethylphenol and Ethyl Pivalate Determined by Partitioning Study

Total Concentration of 3,4-Dimethylphenol, $\times 10^2 M$	Partition Coefficient	$\frac{[DMP_{comp}]}{[DMP_0]}$
0	1.37	—
3.82	1.78	0.30
7.68	2.33	0.70
15.36	3.33	1.44
23.04	4.32	2.17
38.40	6.63	3.86

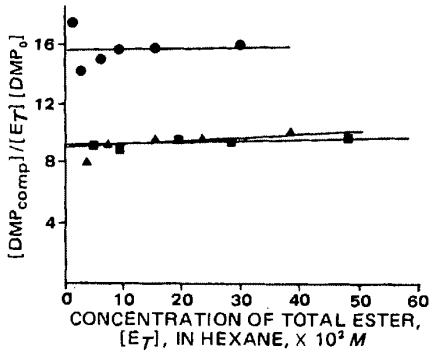

Figure 5—*Plot of $[DMP_{comp}]/[DMP_{free}][E_T]$ as a function of the total ester concentration, $[E_T]$, in hexane; $[DMP_{comp}]$ and $[DMP_{free}]$ represent the concentrations of complexed and free forms of 3,4-dimethylphenol, respectively. ($[DMP_{comp}] = [DMP_T] - [DMP_0]$). Key: ■, ethyl acetate; ●, ethyl myristate; and ▲, ethyl pivalate.*

As seen in Fig. 5, the monohydroxy compound forms only a 1:1 complex with the esters. The stability constants calculated from Fig. 5 are given in Table VII. The results of this study substantiate the conclusion that the diffusion of 4-hexylresorcinol through ethylene–vinyl acetate copolymers involved the formation of 1:1 and 1:2 complexes between the drug and the vinyl acetate portion of the copolymers.

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High-Performance Liquid Chromatographic Analysis of Chemical Stability of 5-Aza-2′-deoxycytidine

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Abstract □ The chemical stability of 5-aza-2′-deoxycytidine (I) in acidic, neutral, and alkaline solutions was analyzed by high-performance liquid chromatography. In alkaline solution, I underwent rapid reversible decomposition to *N*-(formylamidino)-*N*′-β-D-2-deoxyribofuranosylurea (II), which decomposed irreversibly to form 1-β-D-2′-deoxyribofuranosyl-3-guanylurea (III). The pseudo-first-order rate constants for this reaction were determined. The decomposition of I in alkaline solution was identical to that reported previously for the related analog, 5-azacytidine. However, in neutral solution (or water), there was a marked difference in the decomposition of I and 5-azacytidine. The same decomposition products were formed from 5-azacytidine in neutral solution as in alkaline solution. However, in neutral solution, I decomposed to II and three unknown compounds that were chromophoric at 254 nm. Compound I was most stable when stored in neutral solution at low temperature.

Keyphrases □ 5-Aza-2′-deoxycytidine—analysis of chemical stability using high-performance liquid chromatography □ Antileukemic agents—5-aza-2′-deoxycytidine, analysis of chemical stability using high-performance liquid chromatography □ High-performance liquid chromatography—analysis of chemical stability of 5-aza-2′-deoxycytidine

5-Aza-2′-deoxycytidine (I), a nucleoside antimetabolite, is a very active antileukemic agent in mice (1, 2) and a potent cytotoxic agent against neoplastic cells *in vitro* (2, 3). This antimetabolite is related to 5-azacytidine, an agent currently used in the clinical treatment of acute leukemia (4).

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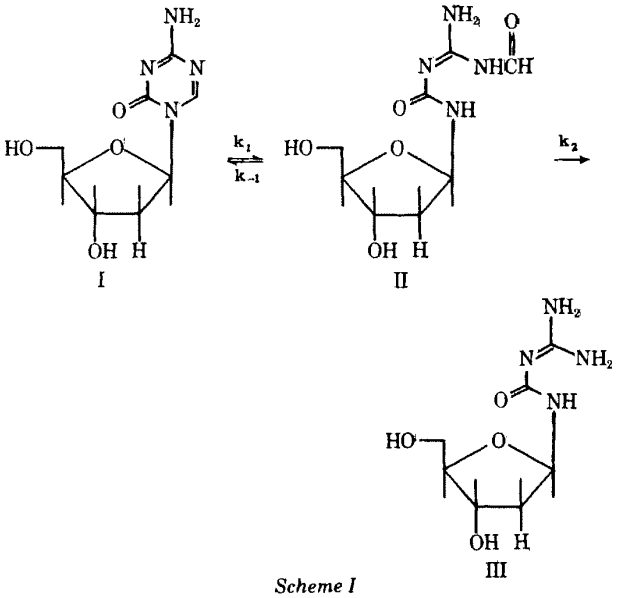

Scheme I

BACKGROUND

One major problem encountered in the clinical formulation of 5-azacytidine is its chemical instability, leading to solutions of decreasing potency on storage. The chemical stability of 5-azacytidine was first studied by Píthová *et al.* (5), who demonstrated that in alkaline solution the triazine ring of 5-azacytidine opens and loses a formyl group to form 1-β-D-ribofuranosyl-3-guanylurea. These workers proposed that the intermediate compound in this reaction was *N*-(formylamidino)-*N'*-β-D-ribofuranosylurea, but they were unable to isolate it using paper chromatography because of its chemical instability. Beisler (6), using high-performance liquid chromatography (HPLC), isolated and identified this intermediate compound and showed that it could be partially converted back to 5-azacytidine.

The chemical decomposition of I at alkaline pH (Scheme I) presumably follows the same reaction steps as described previously for 5-azacytidine. In alkaline solutions, I undergoes a reversible hydrolytic reaction to form *N*-(formylamidino)-*N'*-β-D-2-deoxyribofuranosylurea (II), which, by the irreversible loss of the formyl group, forms 1-β-D-2'-deoxyribofuranosyl-3-guanylurea (III).

The present study investigated the decomposition of I in acidic, neutral, and alkaline solutions and at different temperatures to illustrate the chemical stability of this compound.

EXPERIMENTAL

5-Aza-2'-deoxycytidine (I) was synthesized[1] by modification of an earlier method (7).

Method of Analysis—HPLC[2] was performed with a variable-wavelength detector by monitoring at 220 or 254 nm. Analytical and preparative work was accomplished with a 300 × 3.9-mm i.d. commercially packed octadecylsilane column[3], which was eluted at a flow rate of 2 ml/min with 10 m*M* potassium phosphate buffer (pH 6.8). For analytical and preparative work, 20- and 500-μl injector loops, respectively, were used.

Stock solutions of 10 m*M* I in water were stored at −70°. Aliquots of this solution were thawed quickly and stored at 1–2°. The following buffers were used for the pH stability studies: phosphoric acid, pH 2.2; phosphoric acid–potassium phosphate, pH 3.2; sodium acetate buffer, pH 4.5 and 5.7; potassium phosphate buffer, pH 6.4 and 7.0; and sodium borate, pH 8.5, 9.2, 9.8, and 10.4. Compound II was prepared by addition of 20 μl of 0.5 *M* sodium borate, pH 10.4, to a 1.0-ml solution of 10 m*M* I. The mixture was incubated for 2 min at 24° and then neutralized with 20 μl of 1.0 *M* KH_2PO_4, and a 500-μl sample was injected onto the column for isolation of II.

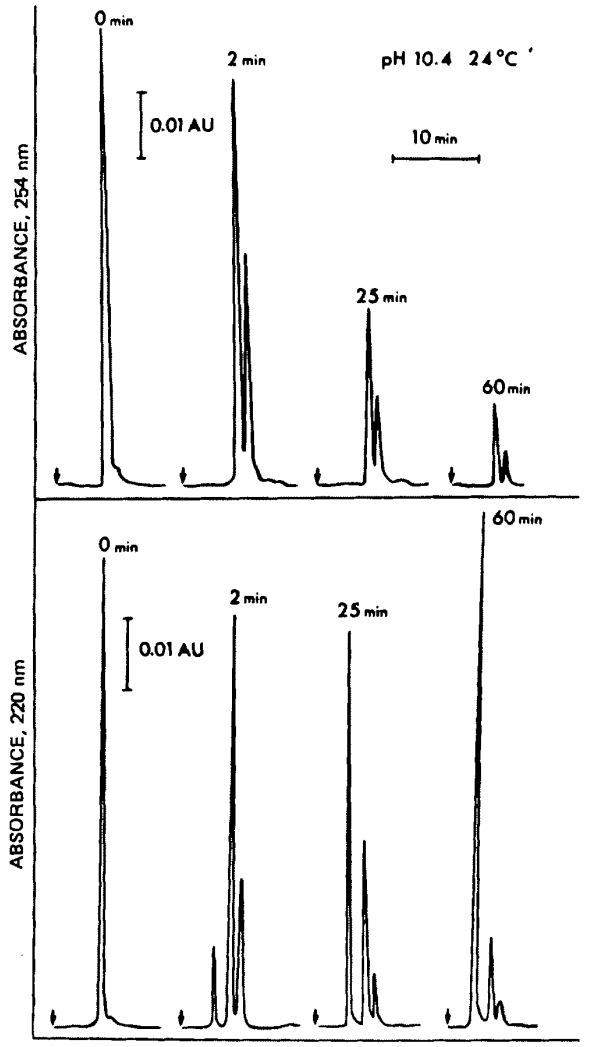

Figure 1—*Decomposition of I at pH 10.4 and 24°. Compound I (1.0 m*M*) was dissolved in 10 m*M *borate buffer (pH 10.4). At the indicated times, an aliquot of the solution was neutralized with phosphate buffer and analyzed by HPLC at 254 and 220 nm.*

Kinetic Experiments—A series of tubes containing 10 μl of 0.5 *M* stock sodium borate buffer were incubated at 37° for 10 min. Then 100 μl of isolated II (in 10 m*M* potassium phosphate, pH 6.8) was added to each tube to start the reaction. At timed intervals, an aliquot was withdrawn, and 20 μl was injected onto the column. The absorbance at 220 nm was recorded, and the relative concentration of the observed degradation products was characterized by peak heights. Similar experimental procedures were followed for the kinetic study on 5-azacytidine[4].

RESULTS

The decomposition of I at pH 10.4 and 24° as determined by HPLC is shown in Fig. 1. Measurement of the absorbance of the column eluate at 254 nm initially showed a single major peak (I) with a t_R value of 5.5 min. With time, a second peak (II) appeared with a t_R value of 6.6 min. Both peaks showed a gradual decrease in peak size with time. At 220 nm, the column eluate initially showed a single major peak (I) with a t_R = 5.5 min. With time, peak II (t_R = 6.6 min) and peak III (t_R = 3.5 min) became evident. At 60 min, most of I decomposed to peak III.

[1] By Dr. A. Piskala, Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Science, 16610 Prague 6, Czechoslovakia.

[2] Model 110 pump and Hitachi variable-wavelength detector, Altex Scientific, Berkeley, Calif.

[3] μBondapak C_{18}, Waters Associates, Framingham, Mass.

[4] Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md.

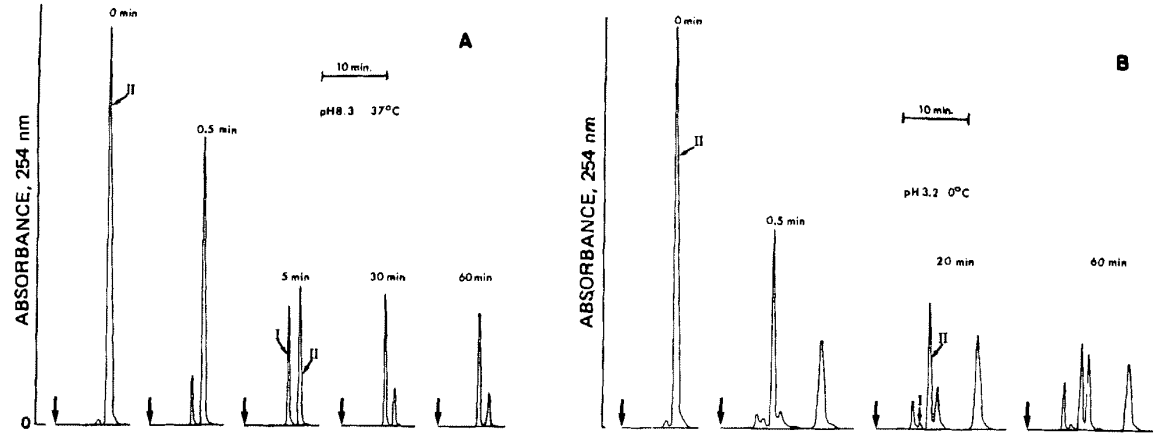

Figure 2—*Conversion of II to I at pH 8.3, 37° (A), and to unidentified compounds at pH 3.2, 0° (B). Peak II was isolated by HPLC and placed in 20 mM potassium phosphate buffer (at final pH 8.3 or 3.2). At the indicated times, aliquots of this solution were analyzed by HPLC.*

When II was isolated and placed at pH 8.3 and 37°, I appeared rapidly, indicating that II could be converted to I (Fig. 2A). However, when II was placed in an acidic solution (pH 3.2), it did not produce I; three unidentified peaks at 254 nm were observed. The latter reaction was very rapid, even at 0° (Fig. 2B).

The decomposition of I and 5-azacytidine when stored in water and in pH 7.4 phosphate buffer at 24° for 24 hr is compared in Fig. 3. Absorbance measurements at 254 nm of the column eluate showed five major peaks for I and two major peaks for 5-azacytidine. At 220 nm, the column eluate showed seven major peaks for I and three major peaks for 5-azacytidine.

The decomposition rates of I in acidic, neutral, and basic solutions at 24 and 37° are shown in Figs. 4A and 4B, respectively. Compound I decomposed more rapidly at pH 2.2, 8.5, and 9.2 than at pH 5.7, 6.4, and 7.0. The decomposition rate of I was the slowest at pH 7.0. At pH 2.2 a peak with the same t_R value (2.3 min) as 5-azacytosine appeared on the chromatogram. The decomposition rate of I was very temperature dependent. For example, at pH 7.0, the decomposition rate was about sevenfold greater at 37 than at 24°.

A kinetic study at pH 8.1 and 9.5 was then carried out with II as the starting substance. According to Scheme I, the pseudo-first-order rate constants k_1, k_{-1}, and k_2 were determined using the following differential

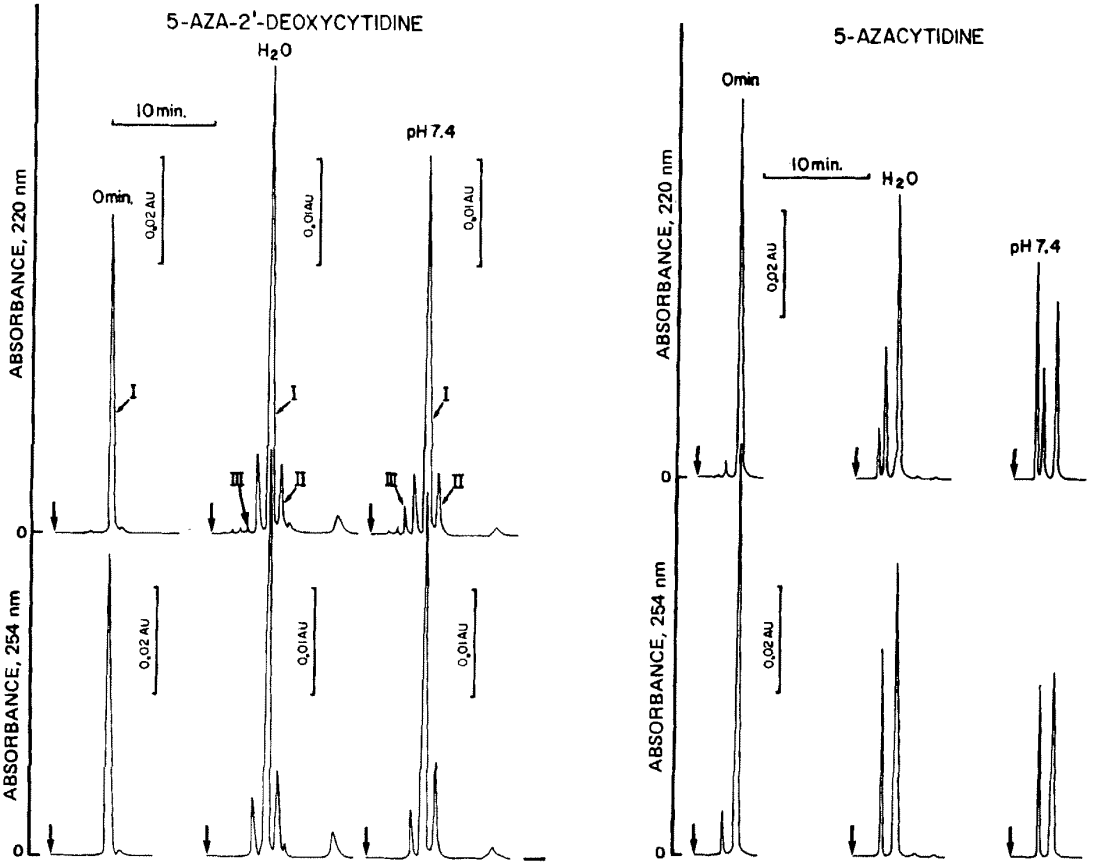

Figure 3—*Decomposition of I and 5-azacytidine in pH 7.4 phosphate buffer and water. 5-Azacytidine (0.5 mM) and I (0.5 mM) were dissolved in 10 mM potassium phosphate (pH 7.4) and water and incubated at 24°. At the indicated times, aliquots of these solutions were analyzed by HPLC at 254 and 220 nm.*

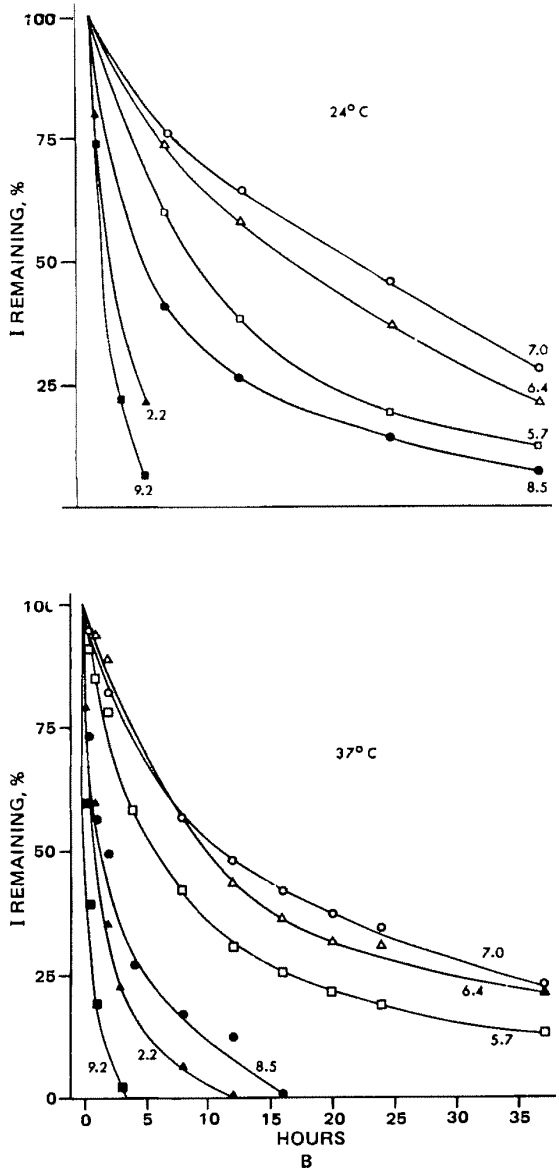

Figure 4—*Effect of pH and temperature on the stability of I. Compound I (1.0 mM) was dissolved in 10 mM of the appropriate buffer with the indicated pH and incubated at either 24° (top) or 37° (bottom). At the indicated times, an aliquot of the solution was neutralized with phosphate buffer and analyzed by HPLC.*

equations (8):

$$I_t = k_{-1} II_0 \left(\frac{1}{B-A} e^{-At} + \frac{1}{A-B} e^{-Bt} \right) \quad \text{(Eq. 1)}$$

$$II_t = II_0 \left(\frac{k_1 - A}{B-A} e^{-At} + \frac{k_1 - B}{A-B} e^{-Bt} \right) \quad \text{(Eq. 2)}$$

$$III_t = II_0 \left(1 - \frac{k_2(k_1 - A)}{A(B-A)} e^{-At} - \frac{k_2(k_1 - B)}{B(A-B)} e^{-Bt} \right) \quad \text{(Eq. 3)}$$

Here A and B are roots of the following quadratic equation taken with the reverse signs:

$$X^2 + X(k_1 + k_2 + k_{-1}) + k_1 k_2 = 0 \quad \text{(Eq. 4)}$$

The rate constants thus obtained are presented in Table I and were used to generate time–concentration profiles for I–III. Normalized experimental data obtained by HPLC were superimposed on these generated time–concentration profiles (Fig. 5). Similar procedures were used for the kinetic study of 5-azacytidine, and the results are shown in Table I and Figs. 6 and 7.

Table I—Rate Constants for the Alkaline Decomposition of I and 5-Azacytidine at 37°

Compound and pH[a]	Pseudo-First-Order Rate Constant, min^{-1}		
	k_1	k_2	k_{-1}
5-Azacytidine, 9.5	2.0×10^{-1}	2.2×10^{-1}	6.0×10^{-1}
5-Azacytidine, 8.1	1.7×10^{-2}	2.0×10^{-2}	5.6×10^{-2}
I, 9.5	2.2×10^{-1}	2.8×10^{-1}	7.5×10^{-1}

[a] The pH value was determined at 37° in a separate tube by mixing one volume of 0.5 *M* stock sodium borate buffer with 10 volumes of 10 m*M* potassium phosphate (pH 6.8).

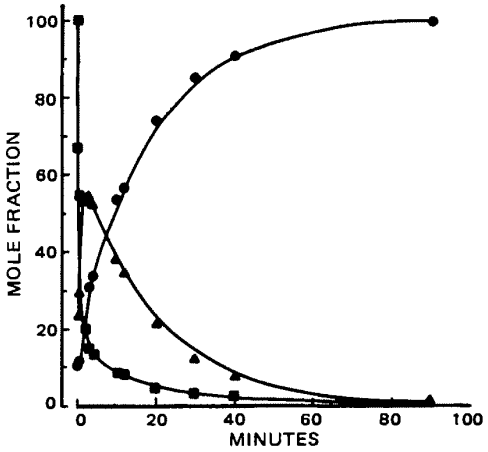

Figure 5—*Time–concentration profile of decomposition for I at pH 9.5 and 37°. Key: ▲, normalized data for I; ■, normalized data for II; and ●, normalized data for III. Solid lines were generated by computer for ▲, ■, and ● using rate constants determined independently.*

DISCUSSION

The hydrolysis of 5-azacytidine in alkaline solution and water results in the opening of the triazine ring between C-6 and N-1 to form *N*-(formylamidino)-*N'*-β-D-ribofuranosylurea in a reversible reaction; when this latter compound loses the formyl group, it irreversibly forms 1-β-D-ribofuranosyl-3-guanylurea (5, 6). By using quantum chemical cal-

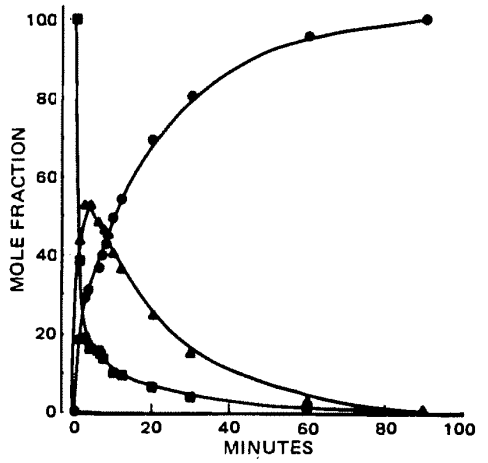

Figure 6—*Time–concentration profile of decomposition for 5-azacytidine at pH 9.5 and 37°. Key: ▲, normalized data for 5-azacytidine; ■, normalized data for N-(formylamidino)-N'-β-D-ribofuranosylurea; and ● normalized data for 1-β-D-ribofuranosyl-3-guanylurea. Solid lines were generated by computer for ▲, ■, and ● using rate constants determined independently.*

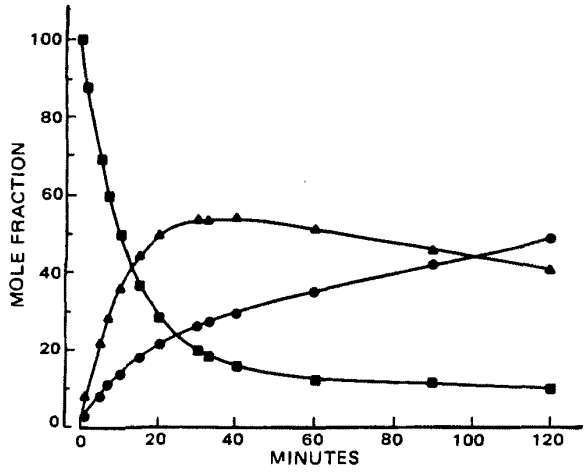

Figure 7—*Time–concentration profile of decomposition for 5-azacytidine at pH 8.1 and 37°. Key: ▲, normalized data for 5-azacytidine; ■, normalized data for N-(formylamidino)-N′-β-D-ribofuranosylurea; and ●, normalized data for 1-β-D-ribofuranosyl-3-guanylurea. Solid lines were generated by computer for ▲, ■, and ● using rate constants determined independently.*

culations, it was shown (5) that the electron density at C-6 of 5-azacytosine is much lower than cytosine, making this position more susceptible to nucleophilic attack by a hydroxyl ion.

From the structural similarity of 5-azacytidine and I, it would seem that I would decompose in alkaline solution according to Scheme I. This study supports this reaction scheme in alkaline solution. As shown in Fig. 1, the decomposition of peak I (t_R = 5.5 min) at pH 10.4 produced peak II (t_R = 6.6 min) when the column eluate was monitored at 254 nm. Peak III was not observed on this chromatogram because guanylurea derivatives are nonchromophoric at 254 nm (5, 6).

However, at 220 nm, peak III (t_R = 3.5 min) became apparent following the decomposition of II to III. When peak II was isolated, it had the same UV_{max} of 238 nm as reported previously (6) for *N*-(formylamidino)-*N*′-β-ribofuranosylurea. The hydrolysis of I in alkaline solution produced an initial increase in absorbance[5] at 238 nm, supporting the formation of II, since the formylguanylurea derivative has a much higher extinction coefficient than 5-azacytosine derivatives at this wavelength (6). The conversion of II to I (Fig. 2A) indicates that this reaction is reversible, which is in agreement with other observations (5, 6) for 5-azacytidine.

There were marked differences in the decomposition of I and 5-azacytidine in phosphate buffer (pH 7.4) and water (Fig. 3). In these solvents, 5-azacytidine decomposed to form formylguanylurea and guanylurea derivatives, as reported previously (6). However, the decomposition of I in phosphate buffer or water was very different from that of 5-azacytidine as shown by the presence of five (254 nm) and seven (220 nm) peaks on the chromatograms of I. Two of these peaks represent I and II, whereas the other peaks were not identified. These unidentified peaks could also be produced rapidly by placing II at pH 3.2 (Fig. 2B), even at 0°. These unidentified compounds were not the intermediates that eventually formed III, since they were not converted to III when placed in alkaline buffer, as would happen to I and II under the same conditions.

Determination of the pseudo-first-order rate constants for I and 5-azacytidine at pH 9.5 and 37° demonstrated that both compounds decomposed at comparable rates, although the formylguanylurea derivative for ribose appeared to be slightly more stable than that of deoxyribose (Table I). A 22-fold decrease in the hydroxyl-ion concentration (pH 9.5–8.1) reduced all three rate constants for 5-azacytidine equally by about 11-fold. Lowering the pH from 9.5 to 8.1 also greatly reduced the conversion rate of II to I and to III (Figs. 5 and 8). The kinetic data obtained for I at pH 8.1 did not quite fit in Eqs. 1–3 because apparently some of the decomposition did not follow Scheme I precisely due to the formation of minor unidentified peaks. Thus the rate constants were not determined. These unidentified peaks became increasingly prominent as the pH was lowered, and there was virtually no conversion of II to I at pH <4.6. (Fig. 2B).

[5] R. L. Momparler, unpublished data.

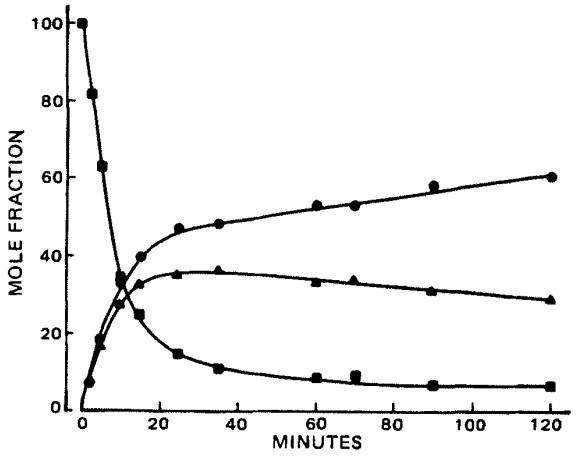

Figure 8—*Time–concentration profile of decomposition for I at pH 8.1 and 37°. Key: ▲, normalized data for I; ■, normalized data for II; and ●, normalized data for III.*

Comparison of the overall stability profile of I in aqueous buffer solution at various pH levels showed that I was most stable at neutral pH and at low temperature (Fig. 4). The instability of I at high pH could be explained on the basis that the opening of the triazine ring (k_1) and the breakage of an amide bond (k_2) is facilitated by a hydroxyl ion. Reduction in pH would slow down both these events and thus stabilize I. However, I became increasing unstable as the pH was gradually reduced below 7.0. This result might be due to the increase in the rate of ring opening as well as to the instability of II in acidic solutions; thus, once ring opening took place and produced II, the latter broke down to the unidentified peaks more rapidly and minimized the reversal back to I (Fig. 2B). In strong acidic solution (with pH <2.2), breakage of the glycolytic bond of I occurred, producing 5-azacytosine[6] as reported previously for I (9) and for 5-azacytidine (5, 10).

The effect of pH and temperature on the chemical stability of 5-azacytidine was studied by Chan *et al.* (11), who observed that this compound is most stable at pH 7.0 and that its rate of decomposition increases in solutions of high or low pH and with temperature.

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[6] Unpublished observations.

Phase I Study of 5-Azacytidine (NSC-102816) Using 24-Hour Continuous Infusion for 5 Days[1,2,3]

Pavel L. Lomen,[4,5] Laurence H. Baker,[4] Gary L. Neil,[6] and Michael K. Samson[4,7]

SUMMARY

The biologic and antitumor activity of 5-azacytidine has been well demonstrated in the past. The drug at present is thought to be primarily cell cycle phase specific. This study was designed to eliminate undesirable side effects (mainly nausea and vomiting) occurring with a bolus dose and to confirm the recent findings of the relative stability of 5-azacytidine's solution with preserved biologic and antitumor activity. In the study we determined that a dose of 150 mg/m^2/day given as a 120-hour continuous iv infusion and repeated at 28-day intervals produced safe, manageable, and reproducible toxicity. The drug was freshly prepared at 4-hour intervals. Eleven courses were administered to seven patients at this dose level and no patient experienced nausea or vomiting. Leukopenia was the major toxic effect. Antitumor activity was shown in one patient with colon cancer and another with American Burkitt's lymphoma.

[Cancer Chemother Rep 59:1123-1126, 1975]

Within the past 10 years of availability, 5-azacytidine has been widely investigated on a clinical as well as on a theoretical pharmacologic basis (1-7).[8] During clinical trials in the US, it has been established that 5-azacytidine has relatively limited use in solid tumors. However, promising results in the treatment of acute leukemias have been demonstrated (3,9). Besides the low remission rate in solid tumors, the major reason for reluctance in using this drug was severe toxicity—nausea and vomiting as well as bone marrow depression.

In all prior trials, the drug was administered iv or sc daily as a single bolus dose (10,11) for several (3-15) days. The major reason for selecting this schedule was the chemical instability of the drug in solution. Recently, however, some studies[9,10] have shown that there are discrepancies between the biologic activity and the chemical stability data. The compound exhibited almost 90% biologic activity (microbiologic assay) even 24 hours after being dissolved. Data from different laboratories[9,11] also have shown that a stability of the drug in solution is dependent on the pH and concentration.

From the presently available pharmacologic and clinical data (12-15) it is obvious that 5-azacytidine has an extremely complicated pattern of metabolism and degradation in vivo and many questions remain to be resolved. Most reports describe 5-azacytidine as being cell cycle phase specific. There are, however, some recent suggestions that the drug has an additional mode of action and that, besides affecting nucleotide metabolism, it acts by impairing cell structures and therefore can attack resting cells (14).

The data mentioned above served as a basis for conducting a phase I study using a 24-hour continu-

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[2]Supported in part by Public Health Service grants CA-07177 and CA-08096 from the National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[3]5-Azacytidine: CAS reg. No. 320-67-2; s-triazin-2(1*H*)-one, 4-amino-1-β-D-ribofuranosyl-.

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[6]Cancer Research Unit, The Upjohn Company, Kalamazoo, Mich.

[7]We wish to thank Mrs. Susan Miedzianowski, the drug team of The Grace Hospital, and Miss Jean Devos for their dedication in dealing with this technically very demanding study.

[8]Broder LE, and Carter SK. Clinical brochure: 5-azacytidine (NSC-102816). Prepared by the NCI, Bethesda, Md, Oct 1970, 25 pp.

[9]Li LH, Buskirk HH, and Hańka LJ. Unpublished data.

[10]Neil GL. Personal communication.

[11]Ben Venue Laboratories (project No. 73-307).

ous infusion for 5 days with these objectives: to eliminate undesirable side effects (mainly nausea and vomiting) and to improve biologic activity of the drug.

MATERIALS AND METHODS

Eight of 11 patients with various disseminated neoplasms were evaluable for toxicity. No patient had previous exposure to 5-azacytidine and all previous chemotherapy and radiotherapy courses were completed at least 4 weeks before entering this study.

Patients had adequate bone marrow function (a peripheral absolute granulocyte count of > 2000 cells/mm^3, a platelet count of > 100,000 cells/mm^3, and a normal bone marrow cellularity), adequate liver function (bilirubin < 1.0 mg/100 ml), and adequate renal function (creatinine of < 1.5 mg/100 ml). Their life expectancy was at least 8 weeks.

The drug was reconstituted with Ringer's lactate solution at a concentration of 2.5 mg/ml. The total daily dose was infused as a 24-hour continuous infusion using an EMS[12] or an IVAC[13] pump and 12-inch intracatheter. The total daily dose was divided into six 4-hour increments, each prepared freshly just prior to the infusion. The course consisted of 5 days of infusion.

There were 11 courses given at the 150-mg/m^2/day level and three courses given at the 170-200-mg/m^2/day level. Courses were repeated beginning on Day 29 or after hematologic recovery. The starting dose was selected after reviewing clinical experience from previous trials where bolus administration was used. The blood counts were done biweekly and continued daily when myelosuppression was detected.

RESULTS

Table 1 shows the diagnoses, previous therapy, and number of courses administered. Table 2 shows a summary of hematologic toxicities. At the 150-mg/m^2 dose level, moderate leukopenia occurred after 18 days, which was relatively late. There was thrombocytopenia in only three courses. This was of moderate degree with an earlier start (12 days) and rapid recovery (3 days). Leukopenia was found in nine courses and in five of these it was selective neutropenia with a relative increase in lymphocyte counts. There were no changes in hemoglobin levels.

Significant toxicity was encountered in patient No. 7 who had been exposed to multiple chemotherapy and total nodal radiation. The patient developed thrombocytopenia and leukopenia on the 5th day of chemotherapy and died of septicemia on the 10th day with platelet and white blood cell counts of 7000 and 700 cells/mm^3 respectively.

There were two patients who showed central nervous system (CNS) abnormalities while receiving 5-azacytidine. The clinical picture was that of somnolence, extreme apathy, and at times disorientation and agitation. There were serial electroencephalogram (EEG) examinations done and diffuse abnormalities were detected. Clinical and EEG findings subsided when the drug was discontinued. No other changes could be found to account for the CNS disturbances. Both myelosuppression and CNS symptoms were clinically easily manageable and did not require premature termination of the course. One patient was treated by hyperalimentation simultaneously and the results of electrolyte determination are difficult to correlate with the chemotherapy. There were no electrolytes abnormalities in the second patient.

There was *no vomiting* and only occasional mild nausea which did not require any medical management. No diarrhea, gastroenteritis, fever, hypotension, or muscle pain was observed.

In patients No. 5 and 7 we noticed antitumor activity that could be easily defined as partial remission. Patient No. 5 had hepatic metastasis from colorectal carcinoma. Hepatic size below the costal margin decreased by 33%. This decrease in liver size could not be shown by liver scan which remained essentially unchanged for 3 months during chemotherapy. Patient No. 7 had > 50% reduction in measurable skin metastasis from a disseminated Burkitt's lymphoma.

In two patients, multiple samples of plasma and urine were analyzed by Dr. Neil using a microbiologic assay. The assay sensitivity, 0.47 μg/ml, probably prevented detection of 5-azacytidine in the plasma since no activity was found in any sample. There was a clearly detectable concentration of free 5-azacytidine in both patients' urine collection. The collections consisted of four 24-hour specimens and the total amount of free drug recovered was 0.89% in the first patient and 0.74% in the second.

DISCUSSION

After the initial enthusiasm 5-azacytidine has been found to be a very toxic drug when administered in a bolus dose. In many reports, the side effects (nausea, vomiting, diarrhea, myelosuppression) were limiting factors in its use. Nausea and vomiting especially were very poorly tolerated by patients. In addition, there were quite significant differences in the clinical studies with respect to

[12]Electro Mechanical Specialties, Inc, Utica, Mich.

[13]IVAC Corp, San Diego, Calif.

TABLE 1.—Diagnoses, previous therapy, and number of courses given

Patient No.	Age (yrs)	Diagnosis	Previous therapy	No. of courses of current therapy
1	45	Hypernephroma (clear cell adenocarcinoma)	Cobalt 60	2
2	59	Squamous cell bronchogenic carcinoma	Methyl-CCNU, vincristine + bleomycin	2
3	67	Lymphocytic lymphoma	Cyclophosphamide, vincristine, adriamycin, prednisone	2
4	28	Lung adenocarcinoma (poorly differentiated)	Cobalt 60 (pleura + brain) 5-Fluorouracil, methyl-CCNU, adriamycin, methotrexate	2
5	64	Sigmoid colon adenocarcinoma	5-Fluorouracil, mitomycin C, vincristine, bleomycin	3
6	51	Small cell lung carcinoma	None	1
7	45	Lymphoma (poorly differentiated, possibly American Burkitt's)	Total nodal irradiation (1/74) Cyclophosphamide, COPP*	1
8	48	Colon adenocarcinoma	5-Fluorouracil, mitomycin C	1

*COPP = cyclophosphamide, vincristine, prednisone, and procarbazine.

TABLE 2.—Summary of hematologic toxicity (leukopenia)

	Dosage group	
	150 mg/m²	170-200 mg/m²
Nadir (cells × 10³/mm³)		
Mean	2.6	1.6
Median	2.3	1.2
Range	0.7-6.1	1.2-1.9
Days to nadir		
Mean	17	21
Median	18	16
Range	10-25	16-26
Days to recovery (>3.0 cells × 10³/mm³)		
Mean	6	3
Median	6	3
Range	2-13	3-4

tumor responses. Some studies found 5-azacytidine to be very disappointing in solid tumors (16) while others were encouraging (17). However, there was a general agreement that the drug is promising in the treatment of acute leukemias (9), again with side effects (nausea and vomiting) as limiting factors.

The findings about the stability of 5-azacytidine[9,11] had prompted us to further investigate the drug in a setting where undesirable side effects would be minimized. Our study showed that using a 24-hour infusion regimen virtually eliminates these side effects while still preserving biologic activity. The solution of the drug that was prepared in exactly the same way as in a clinical setting and was tested by microbiologic assay at different temperatures[14] showed 100% activity after 6 hours. The dose of 150 mg/m² given for 5 days seems to be optimal. We hope that our favorable results will revitalize interest in this promising drug. The preliminary results of our phase II study using the same schedule are favorable.

Recent findings that 5-azacytidine crosses the blood-brain barrier (18), the possibility of using the drug for a local organ perfusion (with the antidote given simultaneously), as well as some most recent indications about the possibility for oral administration (13) certainly open a wide field for further laboratory and clinical studies.

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Phase II Study of 5-Azacytidine (NSC-102816) in the Treatment of Advanced Gastrointestinal Cancer [1,2,3]

Charles G. Moertel, Allan J. Schutt, R. J. Reitemeier, and R. G. Hahn [4]

SUMMARY

5-Azacytidine was administered to 29 patients with advanced gastrointestinal carcinoma; the total dose level ranged between 500 and 750 mg/m² given in 5- or 10-day courses. Although mild to moderate degrees of leukopenia and mild thrombocytopenia were observed, the dose-limiting toxic reaction was severe nausea and vomiting, sometimes associated with diarrhea. Only one transient and partial objective response was observed.

[Cancer Chemother Rep 56:649–652, 1972]

5-Azacytidine was synthesized by Píškala and Šorm in 1964 (1) as an analog of cytidine. It is incorporated into both RNA (2) and DNA (3) and may exert antineoplastic activity by inhibiting DNA, RNA, and protein synthesis. Its inhibitory action is cell-cycle specific, with cells in the S phase showing the greatest sensitivity (4). 5-Azacytidine has shown significant antitumor effect against L1210 mouse leukemia, with little schedule dependency except that single-dose therapy was inferior to continuous or intermittent administration over a 5–9-day period.[5]

In a phase I study by Weiss and associates (5), the most troublesome human toxic effect of 5-azacytidine was nausea and vomiting, which was related to the amount of drug given in each injection. They found that a total dose exceeding 16 mg/kg (approximately 675 mg/m²) would produce a rather marked hematologic depression. Of special pertinence to this paper is that objective remissions were reported in two of six patients with colon cancer.

The present study was undertaken as a phase II investigation of 5-azacytidine in the treatment of patients with advanced gastrointestinal cancer, particularly cancer of the large bowel.

MATERIALS AND METHODS

We selected 29 patients for study, all of whom had histologic confirmation of unresectable adenocarcinoma of the gastrointestinal tract. All were ambulatory outpatients maintaining a reasonable state of nutrition, and all had measurable areas of known malignant disease to serve as an objective indicator of response.

There were 12 men and 17 women. Their mean and median age was 55 years with a range of 31–72 years. Of the 29 patients, 27 had primary cancers in the large bowel, one had a primary pancreatic cancer, and one had a primary gastric cancer.

Fourteen patients had had no previous treatment of their advanced cancer, 15 had had previous chemotherapy (primarily with 5-fluorouracil [table 1]), and five had had previous radiation therapy. No patients, however, had had either radiation therapy or chemotherapy

[1] Received Apr 10, 1972; revised Aug 2, 1972.

[2] Supported by contract NIH 70-2066 from the Division of Cancer Treatment (DCT), National Cancer Institute (NCI), National Institutes of Health.

[3] 5-Azacytidine was supplied by the Cancer Therapy Evaluation Branch, DCT, NCI. Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are listed in the List of Compounds at the end of the text.

[4] Division of Gastroenterology and Internal Medicine (Drs. Moertel, Reitemeier, and Schutt) and Division of Clinical Oncology and Internal Medicine (Dr. Hahn), Mayo Clinic and Mayo Foundation, Rochester, Minn.

[5] Broder LE, and Carter SK. Clinical brochure: 5-azacytidine (NSC-102816), Oct 1970. Prepared by the NCI, Bethesda, Md.

TABLE 1.—Previous treatment experienced by patients in the 5-azacytidine study

Prior treatment	No. of patients
None	14
Chemotherapy	15
5-Fluorouracil	15
CCNU	6
NSC-82196	5
Cyclophosphamide	3
Bleomycin	2
Camptothecin	2
BCNU	1
Radiation therapy	5

within 1 month of entry into the study; all had normal leukocyte and platelet counts.

5-Azacytidine was reconstituted in sterile water and administered by rapid intravenous (iv) push immediately thereafter. We employed three dose schedules: the first, a daily injection for 5 days; the second, a daily injection for 10 days; and the third, injections twice daily for 5 days. The total dose for a course, regardless of the schedule, ranged between 500 and 750 mg/m^2. Twenty-two patients were given total doses of 750 mg/m^2 in their first courses; the total doses of three of the remaining seven patients were raised to this level in their second courses.

Patients were routinely given 10 mg of prochlorperazine (Compazine) hypodermically, immediately prior to each injection of 5-azacytidine. Additional prochlorperazine was given throughout the day as needed, either hypodermically or by rectal suppository.

All patients had full hematologic, hepatic, and renal function studies before treatment and on the day following each course of 5-azacytidine. Leukocyte and platelet counts were obtained twice weekly thereafter. All cases were re-evaluated at 5 weeks, and those patients who were physically able were treated again. If a patient had not shown progression at 10 weeks, treatment was continued at 5-week intervals until progression was evident.

RESULTS

Toxic Reactions

Table 2 shows the toxic reactions experienced by our 29 patients to their first course of therapy. Nausea and vomiting were ubiquitous and very troublesome problems. Patients began vomiting between ½ and 3 hours after receiving an injection of 5-azacytidine and continued for periods ranging from 3 to 10 hours. Phenothiazine antiemetics, even when given in very large doses, were not noticeably effective in alleviating these symptoms. Frequently, a flurry of loose stools would accompany the retching. These symptoms occurred only on the days the drug was administered. Most patients lost weight due to these symptoms. The weight loss ranged between 2 and 13 pounds over the 5–10-day course. Several patients required parenteral hydration. One patient refused to continue therapy after experiencing violent retching and diarrhea with the first dose. This patient is not included in any of the other data presented. To put this problem into perspective, we found 5-azacytidine to be a far more potent emetic agent than either NSC-45388 or NSC-82196.

TABLE 2.—Toxic reactions of patients to first course of 5-azacytidine therapy

Toxic reactions	No. of patients
Nonhematologic (29 patients)	
Nausea	28
Vomiting	27
Diarrhea	11
Dermatitis	1
Hematologic (28 patients)	
Leukocyte count < 4000 > 2000 cells	15
< 2000 cells	3
Thrombocyte count	
< 150,000 > 100,000	8
< 100,000 cells	0

We did not observe any stomatitis. One patient had transient and mild maculopapular dermatitis following each of two courses. There were no other late gastrointestinal or mucocutaneous reactions. Of our patients, 68% showed some evidence of hematologic toxicity. This was usually mild and transient, and there was no instance of life-threatening toxicity. The mean nadir of leukopenia was 25.5 days with a range of 18–30 days. The mean nadir of thrombocytopenia was 18 days with a range of 13–21 days. Cumulative hematologic toxicity with repeated courses was not detected.

There were only two possible instances of hepatotoxicity. One patient had a transient rise in his alkaline phosphatase level and the other

patient had a transient, mild rise in his serum glutamic oxaloacetic transaminase level (SGOT). It seemed likely, however, that these effects could have been due to the large doses of phenothiazines we had used in an attempt to alleviate the vomiting.

Changing from one schedule of administration to another was done primarily to aid the patients in tolerating the vomiting problem. Table 3 shows the severity of vomiting the patients experienced with the first course given in the three schedules we tried. The single injection for 5 days was clinically unacceptable. Twelve of 12 patients had severe and disabling vomiting each day of treatment. The 10-day schedule was a little better, but for most patients it just meant they were in trouble for 10 days instead of 5 and they lost a little more weight. The least troublesome schedule was twice daily for 5 days. Although it is doubtful that with a larger number of patients treated with this schedule the vomiting would be any less severe than with the 10-day schedule, at least it is over in half the time.

The degree and pattern of hematologic toxicity seemed comparable between the 5-day single-dose and twice daily methods. Among ten patients initially treated with a total dose of 750 mg/m² over 10 days, only five of them had leukopenia, compared to nine of 11 when this same total dose was given in 5 days. This finding of increased reactions with a shorter total period of administration is comparable to that recorded by Weiss and associates (6). As might be expected, the mean nadir of leukopenia occurred 2½ days later with the 10-day course than with the 5-day course.

TABLE 3.—Severity of vomiting and schedule of administration for 5-azacytidine therapy

	Administration schedule		
Grade of vomiting*	Once daily × 5 days	Once daily × 10 days	Twice daily × 5 days
4	12	5	1
3		3	3
2		2	4
1			2
0		1	1

*0 = No vomiting; 4 = severe and disabling vomiting each treatment day.

RESULTS

The therapeutic results of 28 patients treated with at least one full course of 5-azacytidine are documented in table 4. Our criterion for objective response was a 50% decrease in the product of the longest perpendicular diameters of the most clearly measurable area of known malignant disease in the absence of an increase in any other lesions or the appearance of new lesions. We observed only one partial response which lasted for only 5 weeks. No objective responses were observed at 10 weeks. This therapeutic activity is therefore significantly inferior to that observed with 5-fluorouracil, which we and most investigators have noted produces objective responses lasting for 2 months or longer at about a 20% response rate. If we were to stretch our criteria to include the more equivocal 25% decrease in tumor size, there would be two additional responses at 5 weeks and one of these was maintained for 10 weeks. If we look at other parameters, such as performance status and body weight, the results are equally dismal.

TABLE 4.—Therapeutic results in 28 patients treated with 5-azacytidine

	Patient status at—					
	5 weeks			10 weeks		
Parameter of response	Improved	Stable	Worse	Improved	Stable	Worse
	No. of patients					
Objective response*	1	8	19	—	3	25
Weight change†	1	8	15			
Performance status‡	1	11	16			

*Improved = > 50% decrease in tumor size (*see* text for discussion); stable = no therapeutic response; worse = disease progression.

†Improved = gain > 2.5% of pretreatment weight; stable = ± 2.5% of pretreatment weight; worse = loss > 2.5% pretreatment weight. Weight at 5 weeks could not be determined in four patients.

‡Improved = 20% or greater (Karnofsky scale [9]); stable = 20% (Karnofsky scale); worse = 20% or greater (Karnofsky scale).

DISCUSSION

Although this trial was not controlled by other modes of therapy for patients with gastrointestinal cancer, it is our belief that the severity of nausea and vomiting induced by 5-azacytidine seriously compromises any hope of clinical usefulness. Since we were unable to demonstrate any meaningful clinical activity in 27 adequately treated patients with cancer of the large bowel, and since preclinical studies do not indicate the likelihood that pursuit of alternative dose schedules would be fruitful, it seems doubtful that further investigation of 5-azacytidine for this neoplasm is justified.

LIST OF COMPOUNDS

5-Azacytidine: NSC-102816; CAS reg. No. 320-67-2; *s*-triazin-2(1*H*)-one, 4-amino-1-β-D-ribofuranosyl-

BCNU: NSC-409962; CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-

Bleomycin: NSC-125066

Camptothecin: NSC-100880; CAS reg. No. 25387-67-1

CCNU: NSC-79037; CAS reg. No. 13010-47-4; 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate; Cytoxan

5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8

NSC-45388: CAS reg. No. 4342-03-4; imidazole-4-carboxamide, 5-(3,3-dimethyl-1-triazeno)-; imidazole carboxamide; DTIC

NSC-82196: CAS reg. No. 5034-77-5; imidazole-4-carboxamide, 5-[3,3-bis(2-chloroethyl)-1-triazeno]-; TIC mustard

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Development of an intravenous formulation for the unstable investigational cytotoxic nucleosides 5-azacytosine arabinoside (NSC 281272) and 5-azacytidine (NSC 102816)

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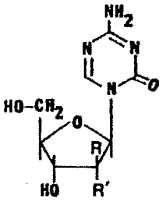
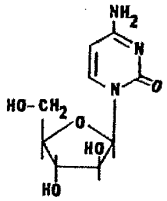
In aqueous solutions 5-azacytosine arabinoside (aza-A) (NSC 281272) exhibits complex and rapid degradation of a type analogous to 5-azacytidine (aza-C) (NSC 102816). Consequently, it is not amenable for use as slow i.v. infusions. This study has determined that both compounds are relatively stable in dry dimethylsulfoxide (DMSO) or dimethylacetamide (DMA). In mixed aqueous-organic solvents, as the water content is reduced the rate of degradation is descreased. Based on these findings, aza-A may be dissolved in DMSO at 100 mg ml^{-1}, sterile filtered, and sealed in ampoules. The contents appear to be adequately stable at 4 °C, and may at the time of use be diluted with water to yield a 70% DMSO solution which retains >90% potency for 24 h at 25 °C and is compatible with commercially available i.v. infusion tubing. The diluted solution may be added in-line to a flowing i.v. vehicle, resulting in a physiologically acceptable solution in which the drug is unstable (t_{90} 2 h). Its short residence time before reaching the bloodstream precludes any significant loss.

5-Azacytosine arabinoside (I, aza-A, NSC 281272) is a nucleoside analogue with antitumour activity related to both 5-azacytidine (II, aza-C, NSC 102816) and cytosine arabinoside (III, ara-C) (Beisler et al 1979).



aza-A, I, R = OH, R′ = H
aza-C, II, R = H, R′ = OH



III, ara-C

Initial data suggest that aza-A exhibits less host toxicity than either aza-C or ara-C (Beisler et al 1979). In man, the use of these antitumour nucleosides is often limited by severe and sometimes dose-limiting nausea and vomiting (Chatterji & Gallelli 1979). Although such toxicity can be controlled effectively by administering the drug as a slow infusion, instability of a drug such as aza-C poses a serious problem (Chatterji & Gallelli 1979). Even when aza-C is infused in lactated Ringer injection, at pH 6·4, which represents conditions at maximum stability in aqueous media, ~10% of aza-C is lost in 2–3 h at room temperature (Chatterji & Gallelli 1979).

Considering that the instability of aza-C is associated with the 5-azacytosine moiety (Notari & DeYoung 1975; Beisler 1978; Benjamin 1979; Benjamin et al 1981), it was expected that similar instability problems would be encountered with aza-A.

We have assessed the relative stability of aza-A and aza-C and designed a formulation allowing slow i.v. infusion.

MATERIALS AND METHODS

Materials

Aza-A and aza-C were used as obtained from the National Cancer Institute (NCI). DMSO was also supplied by NCI in sterile, vacuum sealed vials and was used as received. Spectrophotometric grade DMA was stored over CaO overnight and fractionally distilled under reduced pressure. The first 10 ml was discarded then a constant boiling fraction was collected. Di-(2-ethylhexyl)phthalate was purchased from Aldrich Chemical Co. (Milwaukee, WI) and tri-(2-ethylhexyl)trimellitate was supplied by Cutter Laboratories (Berkeley, CA). Double distilled water

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was used in all experiments. All other chemicals were of reagent grade. The intravenous tubings were from sets i.v. made available by: Travenol Laboratories (Deerfield, IL), #2C006 solution administration set (Lot #6J063R3) and Cutter Laboratories (Berkeley, CA) Saftiset i.v. fat emulsion administration set #808-40 (Lot #NJ1625).

Hplc procedure for determination of aza-A and aza-C

Degradation kinetics of aza-A and aza-C in water and aqueous-organic media were studied at drug concentrations of 5 mg ml^{-1} and 25 °C using a reverse-phase hplc system consisting of a C_{18} μ-Bondapak (~10 μm particle size, 30 × 0·46 cm) column. Aqueous phosphate buffer (0·01 M, pH 6·8) was the mobile phase. Flow rates of 2·0 ml min^{-1} (for aza-A) or 1·5 ml min^{-1} (for aza-C) were employed. Detection was at 254 nm at a sensitivity of 0·1 AUFS. Each drug concentration was diluted 1 : 20 along with the internal standard (0·6 mg ml^{-1} uracil for aza-A and 1·2 mg ml^{-1} cytosine for aza-C) and chromatographed. Samples of 5 μl were injected. For aza-A, the internal standard (uracil), degradation product (IV) and aza-A were eluted with k′ values of 0·5, 1·2 and 1·8, respectively. Similarly for aza-C, the capacity factor for internal standard (cytosine), first degradation compound and the aza-C itself were 0·3, 0·73 and 1·6, respectively. Degradation of aza-A and aza-C were followed qualitatively and quantitatively (via standard curve) constructed from 62·5, 125, 250 and 500 μg ml^{-1} concentrations in distilled water. The plot of ratio of peak areas (aza-A/internal standard) vs concentration was linear (r = 0·9999) regression line being described by: y = 0·01x + 0·001. The inter-assay coefficient of variation (cv) was 1·6%. Under similar conditions, the regression line for aza-C was best described by y = 0·01x + 0·02, r = 0·9997 and cv = 1·5%. The pH of solutions of the drug in distilled water and in mixed solvent systems was measured several times throughout the experiment. In all solutions, the pH appeared to remain constant with the solution in distilled water exhibiting a pH ~5.

Plasticizer assay

The plasticizer di-(2-ethylhexyl)phthalate (DEHP) and tri-(2-ethylhexyl)trimellitate (TEHT) were assayed with a hplc system consisting of a 5 μm Hypersil ODS column (15 × 0·46 cm, 10% carbon load) and using a mobile phase compound of 97 volumes methanol and 3 volumes aqueous 1% acetic acid at a flow rate of 1·0 ml min^{-1}. Sample volumes injected were 20 μl and detection was at 254 nm.

Quantitation employed standard curves and a sensitivity setting of 0·05 AUFs. Standards were prepared in chloroform in the range of 0·1 to 50 μg ml^{-1}. A typical plot of peak height vs concentration for TEHT is given by the regression line: y = 1·6x + 0·7 (r = 0·9997). The inter-assay cv was 3%. For DEHP, the regression line was y = 0·8x + 0·1, r = 0·9998 and cv = 4%. The detection limit of the assay was 0·1 μg ml^{-1}. The k′ values for DEHP and TEHT were 0·5 min and 2·0 min, respectively.

Mixing compatibility

Compatibility and uniform mixing/dilution of the concentrated preparation of aza-A in 70% DMSO (70 mg ml^{-1}) and 70% DMA (60 mg ml^{-1}) in water was assessed in the following way. A sterile commercial i.v. solution of (5% dextrose : 0·45% sodium chloride injection, USP) or sodium chloride was delivered at a rate of 250 ml h^{-1} through a commercial i.v. administration set. The concentrated drug solution was introduced through a side arm tube at the rate of 6 ml h^{-1} using a calibrated infusion pump. The diluted drug solution was subsequently passed through a Whatman filter. Visual observation of the flowing solution at the point of mixing, and examination of the filter, indicated no precipitate formation had occurred.

Equilibrium solubility and long-term stability

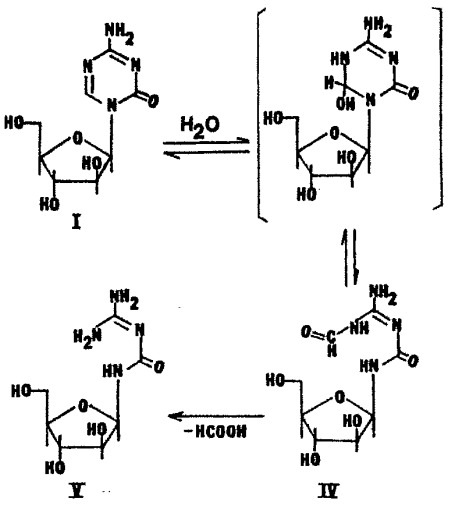
The apparent equilibrium solubility of aza-A was studied in 60, 70 and 100% (v/v) DMA and DMSO in water. Because of drug instability, apparent equilibrium solubilities in the aqueous-organic solution were approximated by hplc analysis of solutions prepared both (a) by agitation of excess solid aza-A with the solvent and (b) by precipitation from supersaturated solutions. In the latter method, the initial concentrations of aza-A in the aqueous DMA and DMSO solutions (before precipitation) were 80 and 160 mg ml^{-1}, respectively. Supersaturated solutions were prepared by dissolution of drug in the organic phase followed by addition of aqueous phase. Each drug solution was first shaken and then centrifuged just before the sampling time and 25 μl of the supernatant was diluted along with the internal standard to 10 ml. A 5 μl aliquot was analysed by hplc. The apparent equilibrium solubility of aza-A in 70% DMSO : 30% H_2O and 70% DMA : 30% H_2O was estimated to be 70 and 60 mg ml^{-1}, respectively (see Fig. 3). Aza-C exhibited a 40 mg ml^{-1} solubility in the 70% DMSO : water system.

Long-term stability studies of aza-A in pure organic solvents were carried out at concentrations

of 100 mg ml^{-1} in DMSO and 85 mg ml^{-1} in DMA. Sealed ampoules were set up at 6, 25, 37 and 50 °C (±1 °C).

RESULTS AND DISCUSSION

Initial studies on the stability of aza-A in aqueous media using hplc demonstrated that it degraded at a comparable rate and by a route analogous to that of aza-C as proposed by Notari & DeYoung (1975) and Benjamin (1979) and substantiated by Beisler (1978). In addition, chromatographic results indicated biphasic kinetics. The data we obtained together with those of the previous workers on aza-C, appear to support a degradation mechanism of the type proposed in Scheme 1, which involves



Scheme 1

Proposed degradation pathway of I in aqueous media.

nucleophilic addition of water to the 5–6 double-bond of the azacytosine moiety accompanied by a proton transfer followed by hydrolysis of IV. It thus appears that the instability could be avoided by use of a solvent that could not participate in one or both of these key steps. Accordingly, the stability of aza-A in DMSO and DMA was investigated since neither solvent should participate in the reactions shown in Scheme 1. Over three weeks, solutions of aza-A in both solvents yielded no evidence of degradation.

The stability at 25 °C of aza-A in solvent systems containing water and either DSMO or DMA in various concentrations was assessed by hplc. Since the degradation kinetics of aza-A are biphasic, the time required for 10% loss (t90) of drug was used as a stability index that usefully reflects drug potency.

The stability of both aza-A (Table 1) and aza-C (Table 2) can be improved by increasing the fraction of organic solvent in the mixed solvent system. The effects of buffer concentration and pH of the mixed aqueous-organic system on the t90 value of aza-A was also examined (Table 1), and at organic solvent

Table 1. Stability characteristics of 5-azacytosine arabinoside (I) at 25 °C in various solvent systems.

Medium	[I] mg ml^{-1}	Measured pH of the solution[a]	t90 (h)
Distilled H_2O	0·25	6·8	1·8
Buffer[b]	0·25	7·0	1·7
5% DMSO in Buffer[c]	5·0	7·0	2·0
10% DMSO in Buffer	5·0	7·1	2·5
25% DMSO in Buffer	5·0	7·4	2·8
5% DMA in Buffer[c]	5·0	7·0	1·9
10% DMA in Buffer[c]	5·0	7·1	2·2
25% DMA in Buffer[c]	5·0	7·4	2·7
50% DMA in Buffer[c]	5·0	8·1	4·5
55% DMA in Buffer[c]	5·0	8·3	6·0
60% DMA in Buffer[d]	5·0	7·4	15·0
60% DMSO in Buffer[d]	5·0	7·8	16·0
70% DMSO in H_2O	5·0	8·0	28·0

[a] In mixed aqueous-organic system, the value represents apparent pH of the final mixture.
[b] Phosphate buffer, 0·01 M, pH 7·0, μ = 0·1 ml^{-1} with NaCl.
[c] Phosphate buffer, 0·01 M, pH 6·8, μ = 0·1 ml^{-1} with NaCl.
[d] Phosphate buffer, 0·03 M, pH 6·0, μ = 0·3 ml^{-1} with NaCl.

Table 2. Stability characteristics of 5-azacytidine (II) at 25 °C in various solvent systems.

Medium	[II] mg ml^{-1}	t90 (h)
Distilled H_2O	5·0	2·0
50% DMSO in H_2O	5·0	7·8
70% DMSO in H_2O	5·0	22·0
70% DMA in H_2O	5·0	19·0

concentrations of >60%, distilled water, rather than buffers, resulted in a slight improvement of the stability index of aza-A. In addition, buffer precipitation, which tended to occur in the organic-aqueous (70:30%, v/v) solution, was avoided. Chromatograms of aza-A in a purely aqueous system and in a aqueous-DMSO (30:70%, v/v) system clearly demonstrated that in the aqueous-organic system, as well as a decreased rate of loss of aza-A, there was

only an insignificant accumulation of IV when compared with the pure aqueous system. A plot of log aza-A remaining as a function of time for both these systems is shown in Fig. 1.

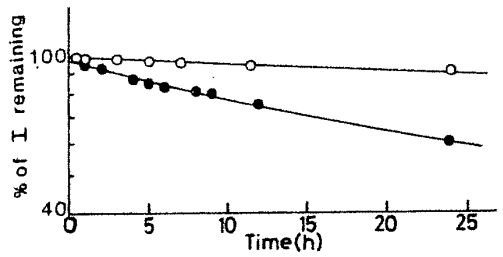


FIG. 1. Semilog plot of degradation of I (aza-A) as a function of time when dissolved in water (●) or 70% (v/v) DMSO-30% water (○) at 25 °C.

While kinetic studies in aqueous solutions at neutral pH exhibited a biphasic degradation profile, in the 70% DMSO : 30% H_2O system the degradation of aza-A appeared to be a simple first-order process in accord with observations that there was no accumulation of IV in such systems. The plot of t90 values as a function of percent (v/v) organic cosolvent is shown in Fig. 2. It is clear that in mixtures with greater than 50% DMA or DMSO the stability of aza-A increased greatly.

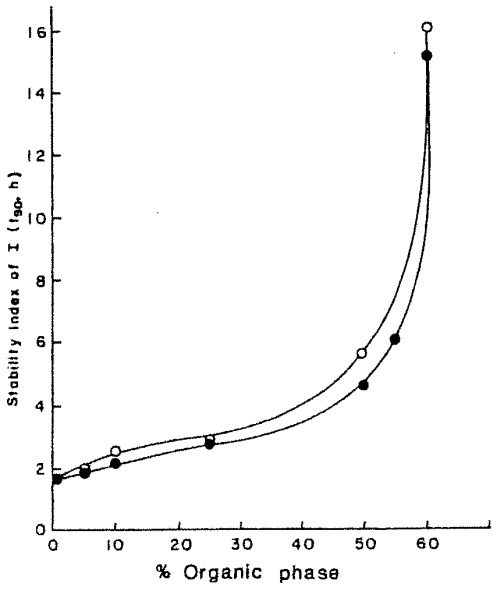


FIG. 2. Plot of stability index of I (aza-A) vs per cent (v/v) organic-aqueous solvent (●, DMA; ○, DMSO) at 25 °C. Phosphate buffer pH 6·8, 0·01 M, ionic strength 0·1 was used as the aqueous phase.

Although the t90 in the cosolvent systems was substantially increased, it was clear that the stability of aza-A in such mixed aqueous-organic solvent systems would not be satisfactory for purposes of manufacturing and distribution as a drug solution. Alternatively, while the stability of aza-A in DMA or DMSO might be suitable for such purposes, the i.v. administration of such solutions would not be pharmaceutically acceptable. In light of the above problems and findings, an alternative strategy was developed.

The approach involved the use of an anhydrous solution of aza-A (in a water miscible organic solvent such as DMSO or DMA) which may be sterilized by filtration, is chemically stable and subsequently diluted at the time of administration. In order to assess the utility of the above approach, the solubility of aza-A as a function of DMSO and DMA concentration in water was evaluated at 25 °C by estimates based on the use of solubility data obtained by hplc monitoring, as a function of time, of the intact drug concentration obtained by agitation of excess drug with the particular solvent system. Values so obtained were compared with values obtained by preparing supersaturated solutions of the drug in the solvent system and monitoring the intact drug concentration as precipitation occurred. Similar asymptotic values of drug solubility were obtained in (Fig. 3) and the solubility data shown in Fig. 4 were obtained. While the solubility of aza-A in

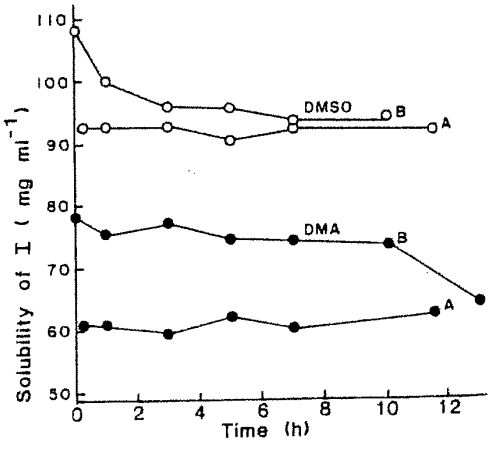


FIG. 3. Determination of apparent equilibrium solubility of I (aza-A) in 70% DMSO–30% water (○) and 70% DMA–30% water (●) as a function of time when the solution was prepared by agitation of excess drug with the solvent (A) or by precipitation from supersaturated solution (B).

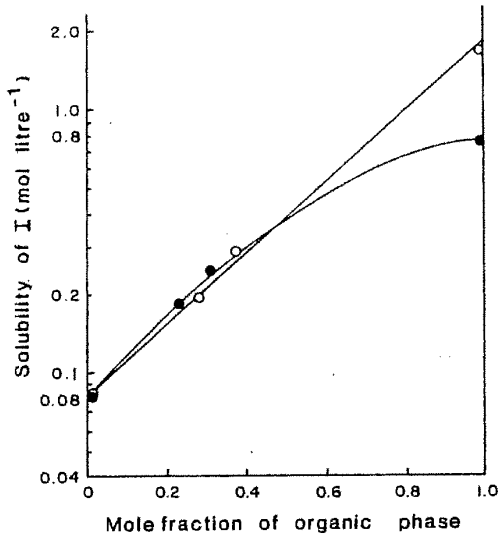


FIG. 4. Plot of apparent saturation solubility of I (aza-A) at 25 °C in solvents composed of various proportions of DMA (●) or DMSO (○) with aqueous phosphate buffer (pH 6, 0·03 M, μ = 0·1).

The compatibility of two samples of commercial tubing with the neat organic solvents and with aqueous-organic solvents was examined. While both systems containing <0·4 mole fraction DMA or DMSO in water were similar, the solubility in DMSO was much greater than in DMA (75 mg ml^{-1} in 70% (v/v) DMA in water and 100 mg ml^{-1} in 70% (v/v) DMSO in water at 25 °C).

were primarily composed of a polyvinyl chloride polymer, they differed in the plasticizers used. In one tubing (Cutter) was tri-(2-ethylhexyl)trimellitate (TEHT) while in the other, (Travenol) it was di-(2-ethylhexyl)phthalate (DEHP). In neat DMA or DMSO, both tubings dissolved in minutes while in 70% organic : 30% water solvents, neither tubing appeared to be physically altered, with both remaining clear and flexible after soaking in the solvent for >24 h.

To ascertain if plasticizer is leached from the tubing, the plasticizer levels in the solvent were determined after various types of exposure of the tubing to the 70% organic : 30% aqueous solvents. The 70% DMSO in water was found to extract less of either plasticizer than 70% DMA in water (Table 3). Also less TEHT is extracted than DEHP by either solvent system. The data suggest that the tubing and solvent of choice in minimizing plasticizer extraction are the TEHT plasticized tubing and the 70% DMSO : 30% water solvent system. Such a combination resulted in <1·5 µg plasticizer extracted from 50 cm lengths of tubing under the most severe treatment investigated.

Table 3. Extraction of plasticizers from intravenous tubing under various treatment conditions at 25 °C.

		Plasticizer extracted[b]			
		30% water–70% DMA		30% water–70% DMSO	
Tubing used (50 cm length)	Treatment[a]	Total (µg)	(µg h^{-1})	Total (µg)	(µg h^{-1})
Travenol	A	1380	345	56·0	14·0
	B	1460	365	64·0	16·0
	C	1800	450	270·0	68·0
Cutter	A	44·0	11·0	0·2	0·05
	B	44·0	11·0	0·6	0·15
	C	112·0	28·0	1·4	0·35

[a] Treatment A = 3·2 ml of liquid flowing through tubing for 4 h. Treatment B = tube filled with 3·2 ml of liquid and allowed to stand for 4 h. Treatment C = tube filled and allowed to stand for 1 h, then removed and tubing refilled with fresh liquid at 1 h intervals for 4 h.
[b] Travenol tubing is plasticized with DEHP, while Cutter uses TEHT.

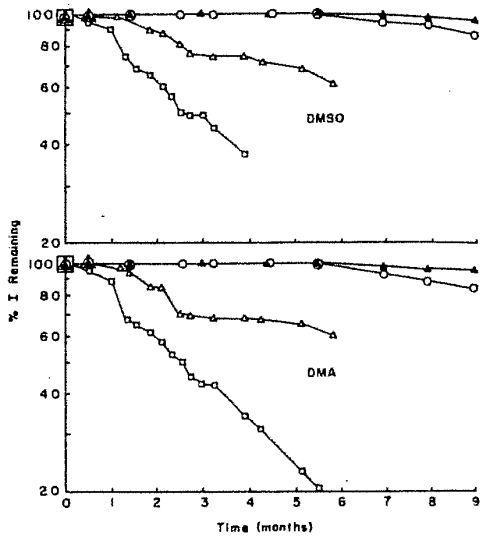


FIG. 5. Log % I (aza-A) remaining as a function of time at various temperatures in DMSO ([aza-A]$_{init}$ = 100 mg ml^{-1}) and in DMA ([aza-A]$_{init}$ = 85 mg ml^{-1}). ▲, 6 °C; ○, 25 °C; △, 37 °C; □, 50 °C.

The ease of mixing and dilution of a 70% DMSO : 30% water solution of aza-A (at 70 mg ml^{-1}) with both saline and 5% dextrose solution was assessed. When the drug solution was infused slowly (6 ml h^{-1}) through Cutt tubing connected to a 20-gauge needle inserted into either of the commercial i.v. vehicles (flowing at a rate of 250 ml min^{-1}), there was no evidence of precipitation nor of any other perceptible incompatibility, and the two solutions appeared to mix readily.

ntravenous tubing
i °C.

r extracted[b]

30% water–70% DMSO	
Total (μg)	(μg h⁻¹)
56·0	14·0
64·0	16·0
270·0	68·0
0·2	0·05
0·6	0·15
1·4	0·35

rough tubing for 4 h.
d allowed to stand for
l for 1 h, then removed
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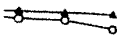

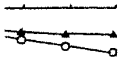

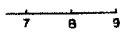

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Long-term studies on the stability of aza-A in neat DMA and DMSO have been undertaken at various temperatures for solutions containing aza-A at concentrations of 85 mg ml^{-1} in DMA and at 100 mg ml^{-1} in DMSO. The results, in Fig. 5, show that the stability of aza-A at 37 and 50 °C is inadequate in either DMSO or DMA with DMSO giving a slightly more stable solution. However, the results at 6 °C indicate a high probability that ≥90% potency of aza-A will be retained for a year or more.

Based on the data thus far obtained relative to stability, solubility, and extent of extraction of plasticizer for i.v. tubing, it appears that DMSO is preferable to DMA as a solvent for aza-A. Less extensive studies similar to those described above for aza-A have also been carried out for aza-C. Results obtained again indicate that the general formulation approach described above for aza-A is similarly applicable for amelioration of the stability/formulation problems presented by aza-C.

Acknowledgement

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Enhancement by Tetrahydrouridine (NSC-112907) of the Oral Activity of 5-Azacytidine (NSC-102816) in L1210 Leukemic Mice [1,2,3]

Gary L. Neil, Thomas E. Moxley, Sandra L. Kuentzel, Rita C. Manak, and Ladislav J. Hanka [4,5,6]

SUMMARY

5-Azacytidine is more active when administered parenterally than orally in the treatment of L1210 leukemic mice. Oral coadministration of tetrahydrouridine, a pyrimidine nucleoside deaminase inhibitor with no intrinsic antitumor activity, greatly increases the oral activity of 5-azacytidine. 5-Azacytidine (or cytotoxic equivalent) blood levels in BDF_1 mice are much higher after oral administration of the 5-azacytidine-tetrahydrouridine combination than when 5-azacytidine is administered alone by the same route. The therapeutic results (L1210 leukemia) achieved with the oral combination are similar to those observed with parenteral 5-azacytidine alone.

[Cancer Chemother Rep 59:459–465, 1975]

5-Azacytidine has shown antitumor activity against transplanted tumors (T-4 lymphoma [1]and L1210 leukemia [1,2]) and spontaneous tumors (AK lymphoma [3]) in mice. It is also an immunosuppressant (4) and an abortifacient (5) in this species. The biochemical mode of action of 5-azacytidine has been actively studied (4,6–9) and clinical trials of its effect in the treatment of human neoplasia are in progress (10–17). 5-Azacytidine has been shown to be an effective treatment of acute leukemia in children (12–14) including tumors resistant to another cytosine nucleoside antimetabolite, cytosine arabinoside (12,13).[7]

We have reported the effects of tetrahydrouridine, a potent inhibitor of pyrimidine nucleoside deaminase, on the oral activity of cytosine arabinoside (18). Although it possesses no antitumor activity itself, tetrahydrouridine markedly increases the oral activity (in L1210 leukemic mice) of cytosine arabinoside by modifying its metabolism and effective absorption. The effects of tetrahydrouridine on the antitumor activity of 5-azacytidine are reported here.

METHODS

The effects of 5-azacytidine and combinations of 5-azacytidine and tetrahydrouridine on the survival of L1210 leukemic mice were studied

[1] Received May 3, 1974; revised Jan 14, 1975; accepted Jan 22, 1975.

[2] This study was supported in part by contract PH43-68-1023 from the Division of Cancer Treatment (DCT), National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[3] Tetrahydrouridine: CAS reg. No. 5627-05-4; 2(1*H*)-pyrimidinone, tetrahydro-4-hydroxy-1-β-D-ribofuranosyl-; THU. Provided by Dr. Arthur R. Hanze (deceased) or Dr. Clifford Y. Peery of The Upjohn Company, Kalamazoo, Mich.

5-Azacytidine: CAS reg. No. 320-67-2; *s*-triazin-2(1*H*)-one, 4-amino-1-β-D-ribofuranosyl-; 5-azaCR. Obtained from the Alfred Bader Chemical Company or through the courtesy of Dr. David G. Martin of The Upjohn Company.

[4] Cancer Research Laboratories, The Upjohn Company, Kalamazoo, Mich.

[5] The authors wish to thank Lynda G. Gray for her technical assistance and Elizabeth L. Clark for assistance in the preparation of this manuscript.

[6] *Reprint requests to:* Gary L. Neil, PhD, Experimental Biology Research Department, Cancer Research, The Upjohn Company, Kalamazoo, Mich 49001.

[7] Cytosine arabinoside: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride; cytarabine; Cytosar; ara-C.

using previously described methods (18) following protocols described by Drug Research and Development, DCT, NCI (19). In all experiments, female BDF_1 (C57BL × DBA/2) mice weighing 18–20 g were used (eight animals/group). Agents were dissolved in sterile 0.9% saline and administered (intraperitoneal [ip] injection or oral intubation) in a constant total volume of 0.2 ml/mouse. Fresh solutions were prepared each day. Animals were inoculated ip on Day 0 with 1×10^6 L1210 cells/mouse and treatment was initiated the following day.

Previous studies (18) have shown that tetrahydrouridine has no intrinsic antitumor effect. Thus, control animals received only oral doses of saline. Where combinations were employed, half of the tetrahydrouridine dose (0.1-ml volume) was administered one-half hour before 5-azacytidine administration and the other half immediately prior to 5-azacytidine administration. Mean survivals, with standard deviations, were calculated for treated and control groups. Percent increases in lifespan (% ILS) were then calculated from these data. Each group of animals was weighed on Days 0 and 5 and the average weight change (g/mouse) was calculated.

Levels of 5-azacytidine (or cytotoxic equivalent) in BDF_1 mouse blood were determined after administration of 5-azacytidine in a dose of 50 mg/kg, ip (experiment 1) or orally (experiment 2) alone or in combination with tetrahydrouridine in a dose of 10 mg/kg by the same route. A divided tetrahydrouridine dose (*see* above) was used. Blood was obtained from the vena cava of anesthetized (Metofane, Pittman Moore Division, Dow Chemical Co., Southgate, Mich) mice (three/time) at various times after 5-azacytidine administration and was stored on ice for less than 3 hours until assay. Blood or a 1:5 dilution in 0.9% saline from each mouse was assayed separately.

The assay employed was a modification of the microbiologic disc-plate assay reported by Hanka et al (1). The assay organism was *Escherichia coli* (ATCC-26) growing in a fully synthetic medium. The synthetic medium consisted of a broth containing KH_2PO_4, 3.0 g; K_2HPO_4, 7.0 g; $MgSO_4$, 0.1 g; $(NH_4)_2SO_4$, 1.0 g; sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$), 1.0 g; glucose, 2 g; tetrahydrouridine, 50 mg; and distilled water to make 1 liter, supplemented with 1.5% agar (high-grade granulated agar, BBL, Cockeysville, Md). Subsequent to autoclaving, the glucose and tetrahydrouridine [8] were added. The seed consisted of 1.0 ml of culture (16–18 hours)/500 ml of agar. Aliquots of 100 ml each were then dispensed into plastic trays (20 cm × 50 cm) (20).

Standard solutions were prepared by dissolving 5-azacytidine (0.5–16 μg/ml) in whole mouse blood (heparinized) or mouse blood diluted (1:5) with 0.9% saline. Standard solutions, in triplicate, and test samples, full-strength blood and 1:5 dilutions in saline in duplicate, were applied to 12.7-mm diameter paper discs (Carl Schleicher and Schuell Co., Keene, NH) on the seeded agar trays. After preincubation at 4° C for 2 hours (which was found to improve sensitivity) trays were incubated overnight at 32° C. Diameters of zones of inhibition were then measured.

Typical standard curves are shown in figure 1. Standard curves were constructed using linear regression analysis on individual assay values; however, only mean values are shown. The sensitivity limits in experiments 1 and 2 were 1.0 and 0.5 μg of 5-azacytidine (or equivalent)/ml of mouse blood, respectively. The concentrations of 5-azacytidine in the test samples were determined using the appropriate standard curve.

The sensitivity of the assay organism to a number of available metabolites or degradation products of 5-azacytidine was also determined. 5-Azacytosine [9] (VI, fig 2), ribose (VIII, fig 2), guanidine (V, fig 2), and biuret (XIII, fig 2)

[8] Previous experiments not described here showed that the addition of tetrahydrouridine (50 μg/ml) to the assay medium increased sensitivity from two- to four-fold, depending on the diluent employed. Similar results have been observed with the sensitivity of *Streptococcus faecalis* (actinobolin-resistant) to cytosine arabinoside (20). No additional increase in sensitivity was observed with concentrations of 100 or 200 μg/ml of tetrahydrouridine. In addition, levels of tetrahydrouridine in blood samples themselves (up to 50 μg/ml) had no effect on the 5-azacytidine values observed.

[9] 5-Azacytosine and 5-azauracil were purchased from Calbiochem, Los Angeles, Calif, and Nutritional Biochemicals Corporation, Cleveland, Ohio, respectively.

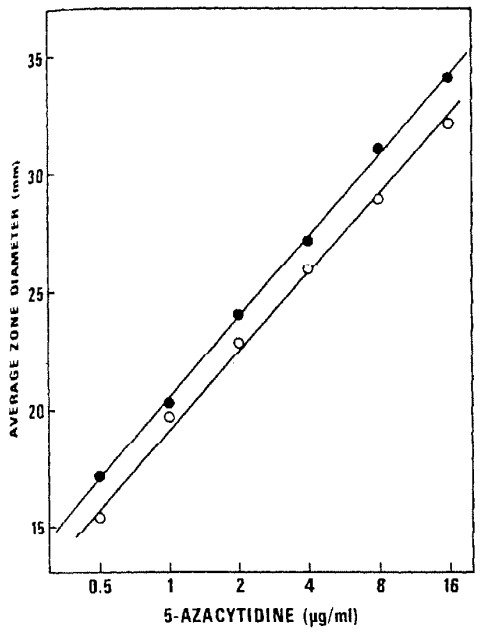

FIGURE 1.—Standard curve for inhibition of *E. coli* by 5-azacytidine. ○ = solutions in mouse blood; ● = solutions in mouse blood diluted (1:5) with 0.9% saline. Pearson's correlation coefficients (r) were 0.9995 and 0.9980 for the mouse blood curve and the diluted blood curve, respectively.

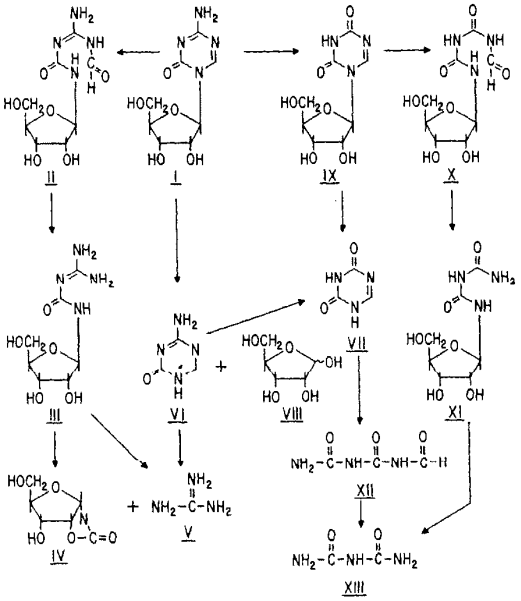

FIGURE 2.—Possible pathways for the degradative metabolism of 5-azacytidine. I = 5-Azacytidine; II = 1-ribofuranosyl-3-(formylguanyl)urea (unstable); III = 1-ribofuranosyl-3-guanylurea; IV = α-D-ribofuro[1',2':4,5]-2-oxazolidone; V = guanidine; VI = 5-azacytosine; VII = 5-azauracil; VIII = ribose; IX = 5-azauridine (unstable); X = ribofuranosylformyl biuret; XI = ribofuranosylbiuret; XII = 1-formylbiuret; XIII = biuret.

were completely inactive at concentrations of 1 mg/ml. 5-Azauracil[9] (VII, fig 2) had approximately one seventh the activity of 5-azacytidine. Thus, all data are expressed as "5-azacytidine (*or* cytotoxic equivalent)."

RESULTS

The effects of tetrahydrouridine on 5-azacytidine therapy of L1210 leukemic mice (single-dose and daily dose schedules) are shown in tables 1 and 2, respectively. 5-Azacytidine used alone was much more effective (especially on a daily dose schedule) when administered ip than when administered orally. For example, daily treatment with 2.5 mg/kg of 5-azacytidine ip was considerably more effective (150% ILS) than treatment with an oral dose of 10 mg/kg/day (83% ILS) (table 2). Using both schedules, ip tetrahydrouridine had little effect, if any, on ip therapy with 5-azacytidine. However, tetrahydrouridine significantly *increased* the effectiveness of 5-azacytidine when both agents were administered orally. Oral therapy (both schedules) with this combination was approximately as effective as therapy with ip 5-azacytidine used alone. Toxicity, as measured by early mortality and weight loss, was also increased when tetrahydrouridine was employed in an oral combination with 5-azacytidine. In figure 3, mean survival is shown as a function of weight change. The weight loss observed at a particular ILS appeared to be independent of the use of tetrahydrouridine. Thus, the therapeutic ratio with the combination does not appear to differ significantly from that observed with 5-azacytidine used alone.

TABLE 1.—Therapy of L1210 leukemic mice with combinations of 5-azacytidine and tetrahydrouridine—single-dose therapy*

5-Azacytidine dose (mg/kg)	Ip administration of—						Oral administration of—					
	5-Azacytidine			5-Azacytidine + tetrahydrouridine			5-Azacytidine			5-Azacytidine + tetrahydrouridine		
	Mean survival (days) ± SD	% ILS	Weight change† (g/mouse)	Mean survival (days) ± SD	% ILS	Weight change† (g/mouse)	Mean survival (days) ± SD	% ILS	Weight change† (g/mouse)	Mean survival (days) ± SD	% ILS	Weight change† (g/mouse)
0 (control)	6.9 ± 0.4		+2.3	6.9 ± 0.4		+2.3	6.9 ± 0.4		+2.3	6.9 ± 0.4		+2.3
12.5	9.8 ± 0.6	42	−0.4	11.3 ± 1.1	64	−1.7	8.0 ± 0.7	16	+1.4	10.6 ± 0.9	54	−1.1
50.0	12.7 ± 0.4	84	−2.3	12.6 ± 0.4	83	−2.8	9.9 ± 0.9	43	−1.0	13.9 ± 1.4	100	−3.4

*Mice were inoculated on Day 0 with 1×10^6 L1210 ascites cells; therapy was started on Day 1; tetrahydrouridine (10 mg/kg) was administered in a divided dose (*see* Methods) by the same route as employed for 5-azacytidine. Control animals received only saline orally. Results are expressed as mean survival times (± SD) and % ILS calculated therefrom.

†Animals were weighed on Days 0 and 5.

TABLE 2.—Therapy of L1210 leukemic mice with combinations of 5-azacytidine and tetrahydrouridine—daily therapy*

5-Azacytidine dose (mg/kg)	Ip administration of—						Oral administration of—					
	5-Azacytidine			5-Azacytidine + tetrahydrouridine			5-Azacytidine			5-Azacytidine + tetrahydrouridine		
	Mean survival (days) ± SD	% ILS	Weight change† (g/mouse)	Mean survival (days) ± SD	% ILS	Weight change† (g/mouse)	Mean survival (days) ± SD	% ILS	Weight change† (g/mouse)	Mean survival (days) ± SD	% ILS	Weight change† (g/mouse)
0 (control)	7.1 ± 0.6		+2.2	7.1 ± 0.6		+2.2	7.1 ± 0.6		+2.2	7.1 ± 0.6		+2.2
2.5	17.8 ± 1.6	150	−3.5	20.3 ± 3.1	186	−4.7	8.6 ± 1.2	21	−0.2	16.2 ± 4.0	128	−4.3
5.0	19.3 ± 2.0	172	−4.1	19.4 ± 1.6	173‡	−4.9	9.6 ± 0.8	35	−2.3	22.7 ± 2.5	220§	−4.7
10.0	19.0 ± 0.8	168‡	−4.7	5.1 ± 1.3	Toxic	−4.7	13.0 ± 1.7	83	−3.6	8.3 ± 2.4	Toxic	−4.9

*Mice were inoculated on Day 0 with 1×10^6 L1210 ascites cells; therapy was started on Day 1 and continued daily for 4 days; tetrahydrouridine (10 mg/kg/day) was administered in a divided dose (*see* Methods) by the same route as employed for 5-azacytidine. Control animals received only saline orally. Results are expressed as mean survival times (± SD) and % ILS calculated therefrom.

†Animals were weighed on Days 0 and 5.

‡Two toxic deaths not included in calculation of mean survival times.

§1/8 Long-term (45-day) survivor.

The effects of tetrahydrouridine on 5-azacytidine mouse blood levels after ip or oral administration of the combination are shown in figure 4. Levels are compared to those found after administration of 5-azacytidine alone. After oral administration of 50 mg/kg of 5-azacytidine alone, maximum 5-azacytidine blood levels were much lower (about 2.1 μg/ml) than after a similar ip dose (about 43 μg/ml). Coadministration of tetrahydrouridine resulted in little increase of maximum 5-azacytidine levels when both agents were administered ip. However, maximum levels were increased approximately three times (to about 6.2 μg/ml) when an oral combination was employed. With both routes, the time during which levels of 5-azacytidine could be detected in blood was increased when tetrahydrouridine was used.

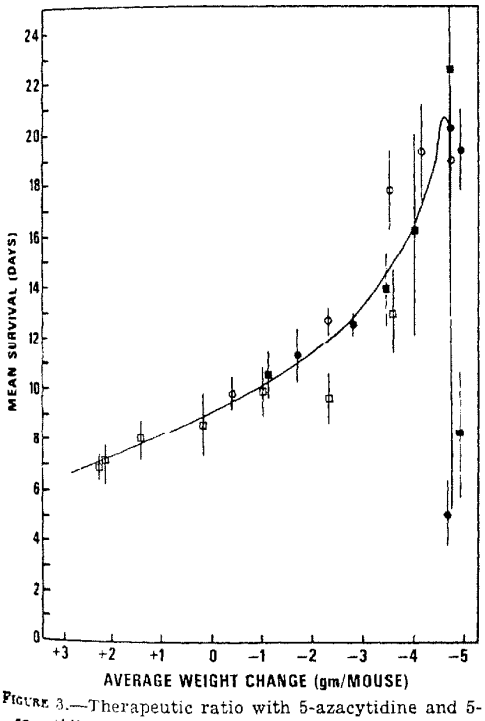

FIGURE 3.—Therapeutic ratio with 5-azacytidine and 5-azacytidine + tetrahydrouridine combinations in the treatment of L1210 leukemic mice. Survival of L1210 leukemic mice (mean ± SD) is shown as a function of weight change (average g/mouse, Days 0–5) observed with various treatments (routes and schedules) with 5-azacytidine or 5-azacytidine + tetrahydrouridine combinations. Data are composite and were obtained from tables 1 and 2. ● = Ip 5-azacytidine + tetrahydrouridine; ○ = ip 5-azacytidine; ■ = oral 5-azacytidine + tetrahydrouridine; □ = oral 5-azacytidine.

DISCUSSION

The effects of tetrahydrouridine on the oral antileukemic activity of cytosine arabinoside have been extensively discussed (18). The results of the present study with tetrahydrouridine and another cytosine nucleoside antimetabolite, 5-azacytidine, are very similar to those of the previous study with cytosine arabinoside.

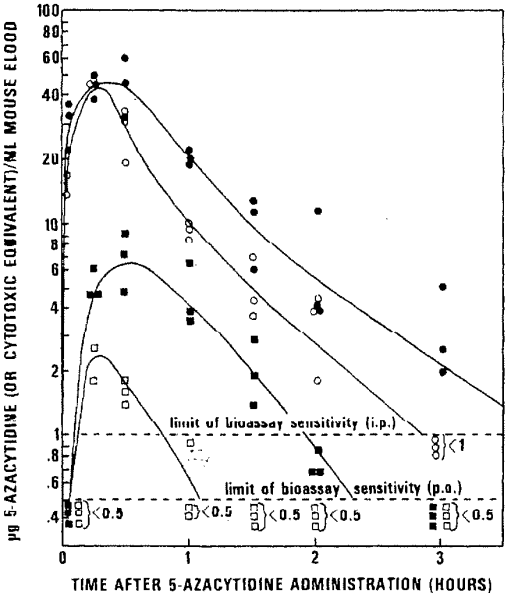

FIGURE 4.—Blood levels of mice receiving 5-azacytidine (or cytotoxic equivalent) after a single administration of 5-azacytidine or a 5-azacytidine + tetrahydrouridine combination. 5-Azacytidine (50 mg/kg) was administered (ip or orally) alone or in combination with tetrahydrouridine (10 mg/kg by the same route). Blood from three mice/time was obtained 5, 15, 30, 60, 90, 120, and 180 min after 5-azacytidine administration. Blood levels were determined with a microbiologic disc-plate assay employing *E. coli* growing in a fully synthetic medium supplemented with tetrahydrouridine (50 μg/ml). ● = Ip 5-azacytidine + tetrahydrouridine; ○ = ip 5-azacytidine; ■ = oral 5-azacytidine + tetrahydrouridine; □ = oral 5-azacytidine.

In both cases, the oral activity of the antitumor agent, administered alone, is much less than that observed parenterally. With both agents, this oral activity is markedly increased by concomitant oral administration of the deaminase inhibitor. Also, in both cases, coadministration of tetrahydrouridine resulted in increased blood levels of the antitumor agent (or cytotoxic equivalent) after oral administration.

In the case of cytosine arabinoside, enzymatic deamination is certainly implicated as a major reason for the low oral activity observed, and the inhibition of this deamination by tetrahydrouridine is apparently the reason for the observed increase in oral activity (18). With 5-azacytidine, it is somewhat more difficult to state unequivocally that this is the case. Whereas the catabolic metabolism of cytosine arabinoside is exquisitely simple (the deamination product, uracil arabinoside [NSC–68928] being the only metabolite observed), the metabolism, including nonenzymatic degradation, of 5-azacytidine is very complex. A representation of some of the possible degradative pathways involved in 5-azacytidine metabolism is shown in figure 2. This scheme is based primarily on the work of Sorm's group (4,21–24) involving in vitro, in vivo, and nonenzymatic (hydrolysis) studies. Compounds I, III, V, VII, XII, and XIII (fig 2) have been identified in the urine of mice treated with 5-azacytidine (4,21). In addition to these metabolites, compounds IV, VI, VIII, and, transiently, II (fig 2) arise in aqueous hydrolysis studies under relatively mild conditions (23). 5-Azacytidine has been shown to be deaminated to 5-azauridine by *Escherichia coli* (24). Recent results of Chabner et al (25) show that 5-azacytidine is deaminated by a human leukemic cell deaminase (the same enzyme which deaminates cytosine arabinoside) and that tetrahydrouridine inhibits this deamination.[10] 5-Azauridine (IX, fig 2) appears (13,14) to be even less stable to hydrolysis than 5-azacytidine and thus it may not be too surprising that it was not observed in mouse urine. Further degradation products of the 5-azauracil moiety (XII and XIII, fig 2) were found however (21). Although these products could arise via pathways (eg, I → VI → VIII, fig 2) other than those involving deamination of 5-azacytidine to 5-azauridine (I → IX, fig 2), this latter route is certainly probable. Indeed the postulated conversion of 5-azacytidine (I, fig 2) to 5-azacytosine (VI, fig 2) is rather unlikely since it might be expected that, like cytidine, it would not be a substrate for any of the known mammalian phosphorylases (26).

Although the deamination product of cytosine arabinoside (uracil arabinoside) has shown no biologic activity and, in particular, does not increase the lifespan of L1210 leukemic mice (unpublished results, these laboratories), the biologic effects of the 5-azacytidine metabolites are less clearly understood. 5-Azauracil (VI, fig 2) has shown some antitumor activity (27) although it has been reported to be inactive in L1210 leukemia. Although no data are available concerning their antitumor activities, 5-azauridine itself and *N*-formylbiuret (compound XII, fig 2) have been shown to possess some, but relatively low, antibacterial activity (28,29).

The lack of any significant effect of tetrahydrouridine on the therapeutic activity of ip administered 5-azacytidine is reasonably consistent with the rather marginal effects of tetrahydrouridine on 5-azacytidine blood levels (especially maximum levels) after administration by that route. Similarly, the enhancement of 5-azacytidine's oral activity by tetrahydrouridine correlates well with the enhanced blood levels observed. However, although the antitumor activity of the oral combination was roughly equivalent to that observed with ip 5-azacytidine alone, blood levels in the former case were clearly inferior to those observed with ip administration. This discrepancy is not easily explained. In our studies, blood was the only tissue assayed for 5-azacytidine activity. Perhaps studies of the kinetics of the disposition of 5-azacytidine in other tissues (particularly lymphoid tissues, ascites fluid, and the tumor itself) would yield more meaningful data for such correlations.

Further studies aimed at determining the importance of nucleoside deamination and its inhibition by tetrahydrouridine on the disposition,

[10] Indeed, of the reactions illustrated in figure 2, the deamination of 5-azacytidine to 5-azauridine is the only one which has definitively been demonstrated to proceed enzymatically.

metabolism, and biologic activity of 5-azacytidine are in progress in our laboratories.

In conclusion, tetrahydrouridine has been shown to increase the effectiveness of another antitumor cytosine nucleoside antimetabolite, 5-azacytidine. The possible clinical use of this inhibitor in combination with agents such as cytosine arabinoside and 5-azacytidine would seem to warrant careful consideration.

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RESEARCH ARTICLES

Kinetics and Mechanisms of Degradation of the Antileukemic Agent 5-Azacytidine in Aqueous Solutions

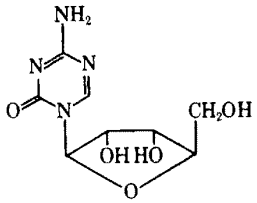
ROBERT E. NOTARI ˣ and JOYCE L. DeYOUNG *

Abstract □ The hydrolytic degradation of 5-azacytidine was studied spectrophotometrically as a function of pH, temperature, and buffer concentration. Loss of drug followed apparent first-order kinetics in the pH region below 3. At pH <1, 5-azacytosine and 5-azauracil were detected; at higher pH values, drug was lost to products which were essentially nonchromophoric if examined in acidic solutions. The apparent first-order rate constants associated with formation of 5-azacytosine and 5-azauracil from 5-azacytidine are reported. Above pH 2.6, first-order plots for drug degradation are biphasic. Apparent first-order rate constants and coefficients for the biexponential equation are given as a function of pH and buffer concentration. A reaction mechanism consistent with the data is discussed together with problems associated with defining the stability of the drug in aqueous solutions. At 50°, the drug exhibited maximum stability at pH 6.5 in dilute phosphate buffer. Similar solutions were stored at 30° to estimate their useful shelflife. Within 80 min, 6×10^{-4} *M* solutions of 5-azacytidine decreased to 90% of original potency based on assumptions related to the proposed mechanisms.

Keyphrases □ 5-Azacytidine—kinetics and mechanisms of degradation in aqueous solutions, effect of pH, temperature, and buffer concentration □ Hydrolysis, 5-azacytidine in aqueous solutions—kinetics and mechanisms, effect of pH, temperature, and buffer concentration □ Antileukemic agents—5-azacytidine, kinetics and mechanisms of degradation in aqueous solutions

The synthesis of 5-azacytidine (I) was reported in 1964 (1). The compound has antimicrobial and antitumor activities (2–4) and is currently being evaluated clinically in the treatment of human leukemias.

The clinical use of this drug makes it desirable to determine kinetic data on its reactivity in aqueous solutions. Such information is especially important in consideration of its relative instability when compared to cytidine itself. Especially notable are the ease with which the triazine ring hydrolyzes and the lability of the sugar–triazine bond (5). Although most hydrolysis products of I have been identified (5), lit-



I

tle has been done concerning the kinetics and mechanism of its degradation.

The purpose of this report is to describe the results of studies on the hydrolysis of I and the related compounds 5-azacytosine (II) and 5-azauracil (III).

EXPERIMENTAL

Analytical Methods—Beer's law plots were constructed for 5-azacytidine, 5-azacytosine, and 5-azauracil in 0.1 *N* HCl using a recording spectrophotometer[1]. Molar absorptivities were 2.36×10^3 for 5-azacytidine at 255 nm; 3.35×10^3, 4.33×10^3, and 5.98×10^3 for 5-azacytosine at 230, 235, and 250 nm, respectively; and 3.67×10^3, 3.80×10^3, and 1.50×10^3 for 5-azauracil at 230, 235, and 250 nm, respectively.

Concentrations of components in mixtures of 5-azacytosine and 5-azauracil were calculated using:

$$10^3[\text{II}] = 0.217A_{250} - 0.088A_{230} \quad \text{(Eq. 1)}$$

$$10^3[\text{III}] = 0.353A_{230} - 0.198A_{250} \quad \text{(Eq. 2)}$$

which were derived from simultaneous equations for total absorbance, *A*, at 230 and 250 nm for mixtures of 5-azacytosine and 5-azauracil in 0.1 *N* HCl. Good agreement was obtained between the results of the assay and the known concentrations in synthetic mixtures.

Mixtures of 5-azacytidine and the two bases were assayed utilizing the differential stability of the components. The absorbance at 255 nm of solutions containing all three components in 0.1 *N* HCl was determined. These solutions were then made 0.05 *N* in sodium hydroxide and allowed to stand at room temperature for 40–50 min. This period was sufficient to degrade 5-azacytidine completely to nonchromophoric products without loss of 5-azacytosine or 5-azauracil. Samples were reacidified with 0.1 *N* HCl, and the absorbances at 235, 250, and 255 nm were determined. The 235- and 250-nm values were used to calculate the concentrations of 5-azacytosine and 5-azauracil:

$$10^3[\text{II}] = 0.234A_{250} - 0.092A_{235} \quad \text{(Eq. 3)}$$

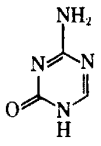
$$10^3[\text{III}] = 0.368A_{235} - 0.267A_{250} \quad \text{(Eq. 4)}$$

After correcting for dilution, the absorbances of the sample before and after treatment with alkali were used to calculate the 5-azacytidine concentration:

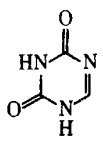
$$\text{I} = \Delta A_{255} / \epsilon_{255} \quad \text{(Eq. 5)}$$

This assay method was shown to be satisfactory using synthetic mixtures.

Kinetics of 5-Azacytosine and 5-Azauracil Hydrolysis in Hydrochloric Acid Solutions—Reaction solutions containing 2×10^{-4} *M* 5-azacytosine or 3×10^{-4} *M* 5-azauracil in 0.1 and 0.25 *N* HCl were placed in constant-temperature baths. Samples of the 5-azacytosine reactions were withdrawn as a function of time and assayed for substrate and 5-azauracil as described previously. The spectra of the 5-azauracil reactions decreased with time, with no evidence of formation of a new chromophore. Rate constants for its loss were calculated directly from absorbance data.



II



III

[1] Cary 15.

Table I—Apparent First-Order Rate Constants (hour^{-1}) for Hydrolysis in Hydrochloric Acid at 70°

Substrate	[HCl], *N*	k_{obs}	k_{23}	k_{25}	k_{36}
5-Azacytosine[a]	0.10	0.376	0.121	0.255	0.0495[b]
	0.25	0.238	0.119	0.119	0.0636[b]
	[HCl], *N*	k_{obs}	k_{12}	k_{13}	k_{14}
5-Azacytidine[c]	0.10	1.98	0.111	0.110	1.77
	0.25	1.05	0.136	0.101	0.810

[a] See Scheme I. Concentration is 2×10^{-4} *M*. [b] Determined from hydrolysis of 5-azauracil (3×10^{-4} *M*). [c] See Scheme II. Concentration is 2×10^{-4} *M*.

TLC of Reaction Solutions of 5-Azacytosine and 5-Azauracil—Samples of the reactions of approximately 0.01 *M* 5-azacytosine and 0.01 *M* 5-azauracil in 0.10 *N* HCl at 70° were taken after about 50 and 100% of the substrate had reacted. These samples were neutralized and spotted along with authentic samples of 5-azacytosine and 5-azauracil on TLC plates coated with 0.25 mm silica gel GF_{254}. The plates were developed in 1-butanol–acetic acid–water (100:10:30) for a distance of about 10 cm, air dried at room temperature, and examined with a shortwave UV lamp.

Kinetics of 5-Azacytidine Hydrolysis in Hydrochloric Acid Solutions—Reaction solutions containing 5-azacytidine in excess hydrochloric acid were prepared as shown in Tables I and II. The absorbance of reactions in solutions less concentrated than 0.1 *N* HCl was determined by diluting aliquots of the reaction with hydrochloric acid to give a final concentration of 0.25 *N* HCl. In these solutions, the UV absorption spectrum of 5-azacytidine decreased with time without the appearance of any new absorbances. Absorbance data were used directly to calculate the apparent first-order rate constants.

In solutions containing 0.1 *N* or stronger hydrochloric acid, there was evidence that a fraction of the 5-azacytidine decomposed to form 5-azacytosine and 5-azauracil. In these cases, the reactions were assayed using the described differential stability method. At 70°, Eqs. 3, 4, and 5 were used to assay for 5-azacytidine, 5-azacytosine, and 5-azauracil, respectively. In the 50° reactions, the differential absorbance due to 5-azacytidine (Eq. 5) was used to calculate the apparent first-order rate constants for loss of substrate.

Kinetics of 5-Azacytidine Hydrolysis in Buffers—Reaction solutions containing 5-azacytidine in excess buffer were prepared as shown in Table III. Solutions were placed in constant-temperature baths; aliquots were removed at appropriate times, chilled, and diluted with an equal volume of hydrochloric acid to give a final concentration of 0.25 *N* HCl. The absorbance of these solutions was measured at 255, 260, and 265 nm. Since absorbance values decreased with time to become zero when the reaction was complete, absorbance data were used directly in the calculation of

Table II—Apparent First-Order Rate Constants for Hydrolysis of 5-Azacytidine[a] at 50° in Hydrochloric Acid

[HCl], *N*	pH[b]	k_{obs}, hr^{-1}
0.80	0.22	0.0595
0.50	0.44	0.0764
0.25	0.73	0.118
0.097	1.12	0.239
0.0097	2.06	1.12
0.005	2.39	1.39

[a] Reactions in 0.80, 0.50, 0.25, and 0.005 *N* HCl contain 4×10^{-4} *M* substrate. Substrate concentration in 0.097 and 0.0097 *N* HCl reactions is 6×10^{-4} *M*. [b] The pH of hydrochloric acid solutions was calculated using activity coefficient values from the literature (6).

Table III—Experimental Conditions and Apparent First-Order Rate Constants for Loss of 6×10^{-4} *M* 5-Azacytidine in the Presence of Buffers in the 7.6–2.6 pH Range at 50°; $\mu = 0.5$

pH	Buffer [NaH_2PO_4]	Buffer [Na_2HPO_4]	Buffer [NaCl]	k_a[a], hr^{-1}	k_b[a], hr^{-1}	A[a]	B[a]
7.59 ± 0.08	0.015	0.15	0.035	15.0	0.74	0.17	0.83
	0.010	0.10	0.19	13.2	0.62	0.17	0.83
	0.005	0.05	0.345	11.2	0.50	0.20	0.80
	0.003	0.03	0.407	10.6	0.44	0.18	0.82
		Unbuffered		9.42[b]	0.37[b]	0.18[c]	0.82[c]
6.52 ± 0.04	0.10	0.10	0.10	5.49	0.29	0.19	0.81
	0.08	0.08	0.18	5.05	0.24	0.21	0.79
	0.05	0.05	0.30	4.38	0.18	0.20	0.79
	0.02	0.02	0.42	3.34	0.12	0.18	0.82
		Unbuffered		2.91[b]	0.076[b]	0.20[c]	0.80[c]
5.42 ± 0.01	0.30	0.03	0.11	5.51	0.41	0.28	0.72
	0.20	0.02	0.24	4.87	0.29	0.25	0.75
	0.10	0.01	0.37	5.19	0.18	0.21	0.79
	0.06	0.006	0.42	5.03	0.13	0.22	0.78
		Unbuffered		5.15[c]	0.065[b]	0.24[c]	0.76[c]
	[CH_3COOH]	[CH_3COONa]	[NaCl]				
5.60 ± 0.04	0.040	0.40	0.10	4.39	0.15	0.26	0.74
	0.024	0.24	0.26	4.83	0.12	0.23	0.77
	0.016	0.16	0.34	4.72	0.099	0.24	0.76
	0.008	0.08	0.42	4.38	0.080	0.25	0.75
		Unbuffered		4.58[c]	0.064[b]	0.24[c]	0.76[c]
4.62 ± 0.04	0.400	0.400	0.100	2.15	0.48	0.60	0.40
	0.240	0.240	0.260	3.04	0.41	0.47	0.53
	0.144	0.144	0.356	3.70	0.37	0.32	0.68
	0.080	0.080	0.420	4.11	0.27	0.32	0.68
		Unbuffered		4.57[b]	0.25[b]	0.23[b]	0.77[b]
				k_{obs}			
3.58 ± 0.02	0.40	0.040	0.460	1.82[d]			
	0.24	0.024	0.476	1.79[d]			
	0.16	0.016	0.484	1.80[d]			
	0.08	0.008	0.492	1.80[d]			
		Unbuffered		1.80[c]			
	[HCOOH]	[HCOONa]	[NaCl]				
2.58 ± 0.06	0.40	0.040	0.460	1.57[e]			
	0.24	0.024	0.476	1.58[e]			
	0.16	0.016	0.484	1.60[e]			
	0.08	0.008	0.492	1.69[e]			
		Unbuffered		1.61[c]			

[a] See text for explanation of these constants and the method used to calculate them. Average of results for two or three wavelengths. [b] Obtained from intercept of plot of *k versus* buffer concentration. [c] Average of values in presence of buffer. [d] It was not feasible to separate the two phases for analysis. The rate constants given were obtained from the first 17% of the reaction. See text for further details. [e] Reaction appears first order over at least two half-lives.

rate constants. In phosphate buffers of pH 7.6 and 6.5, spectra of nonacidified samples were also obtained. At wavelengths greater than 240 nm, there was eventual loss of the UV absorption spectrum. Absorbance data in this region were used directly for the calculation of rate constants.

In two additional experiments, samples of 5-azacytidine (10^{-2} *M*) were reacted in buffer for a sufficient time to ensure that the initial rapid phase of the reaction was completed. Both reactions were conducted at 70°, one in 0.33 *M* phosphate buffer at pH 7.59 for 10 min and the other in 0.04 *M* phosphate buffer at pH 6.52 for 1 hr. Aliquots from each reaction were added to 10 times their volume of hydrochloric acid at 70° to yield a final reaction condition of 0.1 or 0.25 *N* HCl (Table IV). These reactions were then followed at 70° using the differential assay for 5-azacytidine described earlier (Eq. 5).

Evidence for 5,6-Addition to 5-Azacytidine—The UV spectra of 5-azacytidine and 5-azacytosine were recorded at each of three conditions: 0.1 *N* HCl, 0.001 *N* HCl, and pH 6.5 phosphate buffer. Each solution was then altered to match one of the others so that the sequence represents all possible combinations of the three conditions. Precautions were taken to eliminate any changes in absorbance due to passage of time or dilution of sample. In this way, UV spectra for solutions of authentic samples were compared to those that had been previously prepared and recorded at one or more of the other conditions as described under *Results*.

Solutions of 5-azacytidine (6×10^{-5} *M*) and 5-azacytosine (1.5×10^{-5} *M*) were prepared in 0.025, 0.005, and 0.001 *M* aqueous bisulfite previously adjusted to pH 3.67 ± 0.06. A fourth solution of 5-azacytidine in 0.001 *M* bisulfite containing 0.024 *M* NaCl was also prepared. Immediately after dissolution of the substrate, UV spectra were recorded as a function of time.

Spectrophotometric Determination of pKa Values for 5-Azacytosine—The pH of a solution containing 10^{-4} *M* 5-azacytosine in buffer (0.08 *M* acetic acid–0.008 *M* sodium acetate–0.492 *M* sodium chloride) was measured, and the UV spectrum of the solution was obtained. The pH was then adjusted using hydrochloric acid or sodium hydroxide to obtain a series of spectra ranging from the cationic form of 5-azacytosine to the anionic form. To check for degradation, a sample at high pH was adjusted to a previously obtained lower pH value and the two spectra were compared to show that no irreversible changes had occurred.

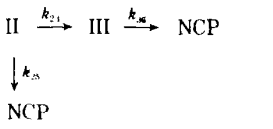
Table IV—Apparent First-Order Rate Constants[a] ($hour^{-1}$) in Hydrochloric Acid at 70° for 5-Azacytidine Compared to Those Obtained when 5-Azacytidine was Previously Reacted in Phosphate Buffer[b]

[HCl], *N*	Unreacted Sample	Phosphate Buffer[b] pH 6.5	Phosphate Buffer[b] pH 7.6
0.10	1.98	1.86	1.95
0.25	1.05	1.07	—

[a] Average of values obtained at two or three wavelengths. [b] See *Experimental* for reaction conditions.

RESULTS

Apparent First-Order Rate Constants in Hydrochloric Acid Solutions—Good first-order plots were obtained when ex-



Scheme I

perimental data were graphed according to:

$$\ln X = \ln X_0 - k_{obs}t \qquad \text{(Eq. 6)}$$

where t is time, X is the concentration of unreacted 5-azacytidine or 5-azacytosine, and X_0 is the initial concentration. When appropriate, data were plotted according to:

$$\ln A = \ln A_0 - k_{obs}t \qquad \text{(Eq. 7)}$$

where A is the absorbance due to unreacted substrate at 255, 260, or 265 nm for 5-azacytidine or at 230 or 250 nm for 5-azauracil. The apparent first-order rate constants for loss of 5-azacytidine and 5-azacytosine (k_{obs}) and 5-azauracil (k_{36}) are listed in Table I.

TLC of Reaction Solutions of 5-Azacytosine and 5-Azauracil in 0.10 *N* Hydrochloric Acid—The 50 and 100%-reacted samples of 5-azacytosine showed faint spots at the same R_f value as an authentic sample of 5-azauracil. The 100%-reacted sample showed no trace of starting material. The 5-azauracil reaction showed a disappearance of the spot due to the uracil without development of any other compounds absorbing UV light. The R_f values obtained were 0.38 for 5-azacytosine and 0.58 for 5-azauracil.

Kinetics of 5-Azacytosine and 5-Azauracil Hydrolysis in Hydrochloric Acid Solutions—The observed spectral changes during the reactions and the results of the TLC experiments pointed to the applicability of Scheme I to 5-azacytosine hydrolysis. Nonchromophoric products are designated as NCP. A value for k_{36} may be obtained directly from 5-azauracil hydrolysis, while k_{23} and k_{25} were obtained from k_{obs} by:

$$k_{obs} = k_{23} + k_{25} \qquad \text{(Eq. 8)}$$

and:

$$k_{23} = fk_{obs} \qquad \text{(Eq. 9)}$$

where f is the fraction of 5-azacytosine that deaminates to 5-azauracil. The method used to calculate f is described below. In the case of a sequential reaction with parallel first-order loss, as in Scheme I, the concentration of III as a function of time is given by:

$$[III] = \{k_{23}[II]_0/(k_{36} - k_{23} - k_{25})\}\{e^{-(k_{23}+k_{25})t} - e^{-k_{36}t}\} \qquad \text{(Eq. 10)}$$

where $[II]_0$ is the initial concentration of 5-azacytosine. The area under a plot of [III] *versus* time is found by integrating Eq. 10 from zero to infinity:

$$(\text{area III})_1 = f[II]_0/k_{36} \qquad \text{(Eq. 11)}$$

where f is the same as in Eq. 9. Similarly, for the simple first-order loss:

$$III \xrightarrow{k_{36}} NCP \qquad \text{(Eq. 12)}$$

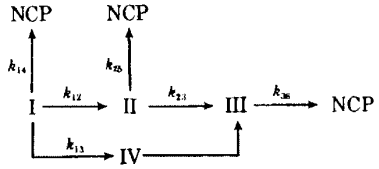
Integrating the equation for III yields:

$$(\text{area III})_2 = [III]_0/k_{36} \qquad \text{(Eq. 13)}$$

Application of Eqs. 11 and 13 to experimental data allows the calculation of f. The concentration of 5-azauracil produced from 5-azacytosine was plotted as a function of time. During formation from 5-azacytosine, the area under this curve was estimated by the method of trapezoids. After the 5-azacytosine was exhausted, the remaining area was calculated from:

$$(\text{area})_{t\to\infty} = [III]_t/k_{36} \qquad \text{(Eq. 14)}$$

where $[III]_t$ is the concentration of 5-azauracil at time t, and k_{36} is the apparent first-order rate constant for 5-azauracil loss under



Scheme II

identical conditions. The sum of the two areas provides the total area, $(\text{area III})_1$. The area under the concentration *versus* time curve for hydrolysis of an equimolar solution of 5-azauracil, $(\text{area III})_2$, was also calculated from Eq. 14. Since $[II]_0 = [III]_0$, Eqs. 11 and 13 may be combined to yield:

$$(\text{area III})_1/(\text{area III})_2 = f \qquad \text{(Eq. 15)}$$

The rate constants obtained are listed in Table I. These were used in an analog computer[2] program to simulate the time course of the various reaction components. A typical plot is shown in Fig. 1, which shows good agreement between observed and computer-generated values.

Kinetics of 5-Azacytidine Hydrolysis in Hydrochloric Acid Solutions—The proposed path for 5-azacytidine hydrolysis in 0.1–0.8 *N* HCl is shown in Scheme II. The apparent first-order rate constants k_{12}, k_{14}, and k_{13} were determined in a manner similar to that used for k_{23} and k_{25}. For example:

$$k_{12} = f(k_{obs}) \qquad \text{(Eq. 16)}$$

where f is the fraction of 5-azacytidine that hydrolyzes to 5-azacytosine. This fraction is obtained using Eq. 15, replacing $(\text{area III})_1$ with the area under the concentration curve for 5-azacytosine produced from 5-azacytidine and replacing $(\text{area III})_2$ with the area starting with 5-azacytosine itself.

Since the values for k_{25}, k_{23}, k_{36}, and the time course for II are all known (Scheme II), they can be employed to define the time course for $[III]_{II}$ (the 5-azauracil arising from 5-azacytosine). The difference between the observed values for III as a function of time, [III], and the expected values, $[III]_{II}$, defines the time course for III arising from IV *via* k_{13}:

$$[III]_{IV} = [III] - [III]_{II} \qquad \text{(Eq. 17)}$$

The fraction of I converted to III by this route was used to calculate the value for k_{13}; this value, in turn, was employed to calculate k_{14} from:

$$k_{14} = k_{obs} - k_{12} - k_{13} \qquad \text{(Eq. 18)}$$

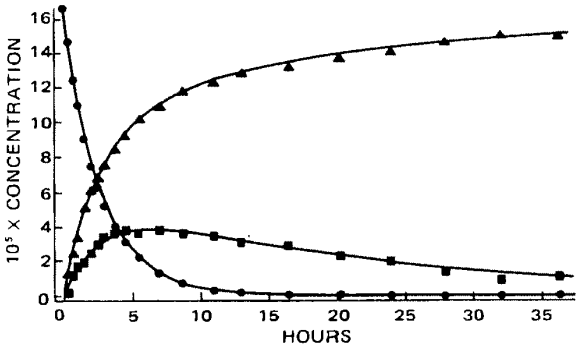


Figure 1—*Time course for 5-azacytosine (●), 5-azauracil (■), and nonchromophoric products (▲) during the hydrolysis of 5-azacytosine in 0.1* N *HCl at 70°. The lines were generated using an analog computer programmed with rate constants listed in Table I.*

[2] EAI, model TR 20.

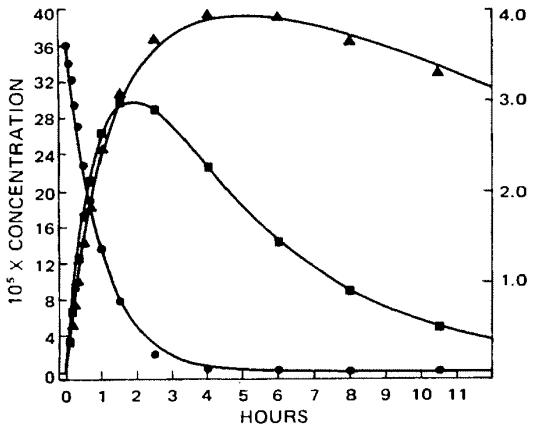


Figure 2—*Time course for 5-azacytidine (●), 5-azacytosine (■), and 5-azauracil (▲) during the hydrolysis of 5-azacytidine in 0.25 N HCl at 70°, showing the agreement between the experimental points and the computer-generated lines. The scale for 5-azacytosine and 5-azauracil is given on the right ordinate.*

These analyses were carried out for the reaction conditions listed in Table I. Typical plots are shown in Fig. 2. The k_{obs} values obtained at 50° are listed in Table II.

Kinetics of 5-Azacytidine Hydrolysis in Buffer Solutions—In buffers at pH 4.6 or higher, the plots of absorbance *versus* time were distinctly biphasic. Acidified samples gave plots similar to Fig. 3, showing a rapid initial decrease in absorbance, followed by a much slower second phase. When the spectra of buffered samples were determined, there was a rapid initial increase in absorbance followed by a slower decrease (Fig. 4).

The biphasic curves were analyzed by feathering the data using commonly employed methods[3]. Treated in this way, each reaction produced two linear first-order plots (Figs. 3 and 4). If the data are normalized relative to the initial absorbance values, a single equation, wherein the algebraic sign for the coefficient A is negative in buffer and positive in acid, may be used to describe the biphasic

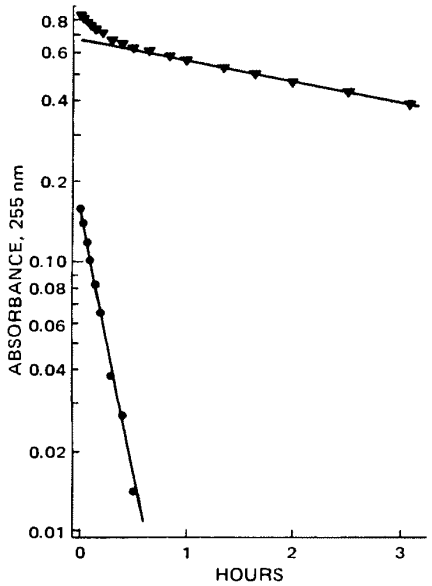


Figure 3—*Absorbance of acidified samples at 255 nm as a function of time (▼) for 5-azacytidine hydrolysis at 50°, pH 6.52, showing lines of slope* k_a *(●) and* k_b *(▼) obtained by feathering.*

[3] For a typical example of this method of data analysis, see Ref. 7.

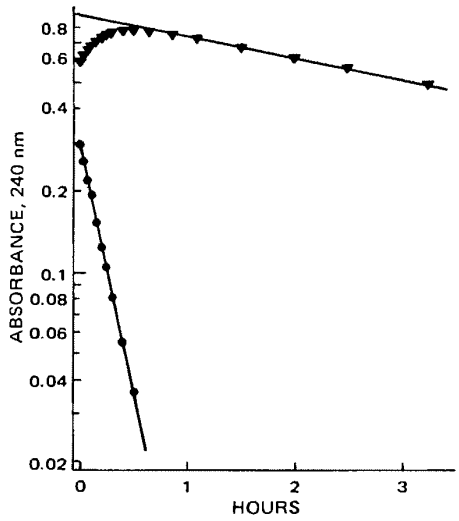


Figure 4—*Absorbance at 240 nm as a function of time (▼) for nonacidified samples of 5-azacytidine at 50°, pH 6.52, showing lines of slope* k_a *(●) and* k_b *(▼) obtained by feathering.*

curves:

$$F(A) = \pm Ae^{-k_a t} + Be^{-k_b t} \qquad \text{(Eq. 19)}$$

where $F(A)$ is the fraction of absorbance remaining; A and $-k_a$ are the intercept and slope of the rapid phase, respectively; and B and $-k_b$ are the intercept and slope of the slow phase, respectively. Rate constants are independent of the pH of analysis. Their values, together with the coefficients in acid, are given in Table III.

Table IV compares first-order rate constants for hydrolysis of 5-azacytidine in hydrochloric acid at 70° to those obtained for 5-azacytidine previously reacted in phosphate buffer. Although the buffer reactions had proceeded into the second phase, the observed rate constants after transfer to hydrochloric acid were the same as those for authentic samples.

At pH 3.58, the plots of ln (A) *versus* time showed definite curvature but were not distinctly biphasic. In fact, the plots were adequately described by a first-order equation over approximately the first half-life. Rate constants, estimated using data representing the first 17% of the reaction, are reported in Table III.

Hydrolysis was apparent first order at pH 2.58, and the rate constants were obtained using Eq. 7 (Table III).

Effect of Buffer on Hydrolysis Rate—Plots of k_a, k_b, or k_{obs}

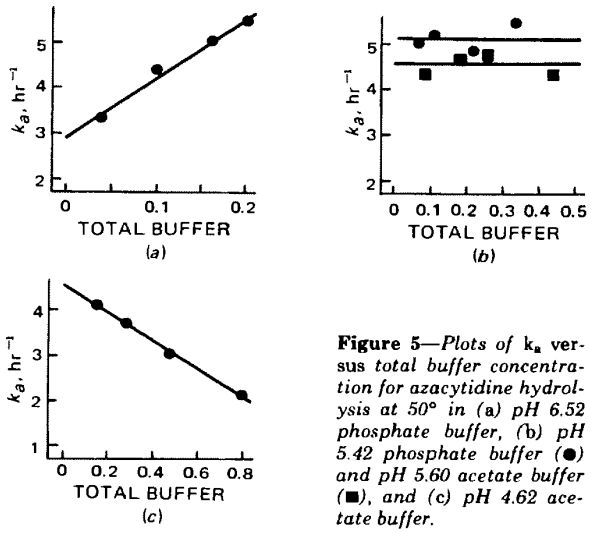


Figure 5—*Plots of* k_a *versus total buffer concentration for azacytidine hydrolysis at 50° in (a) pH 6.52 phosphate buffer, (b) pH 5.42 phosphate buffer (●) and pH 5.60 acetate buffer (■), and (c) pH 4.62 acetate buffer.*

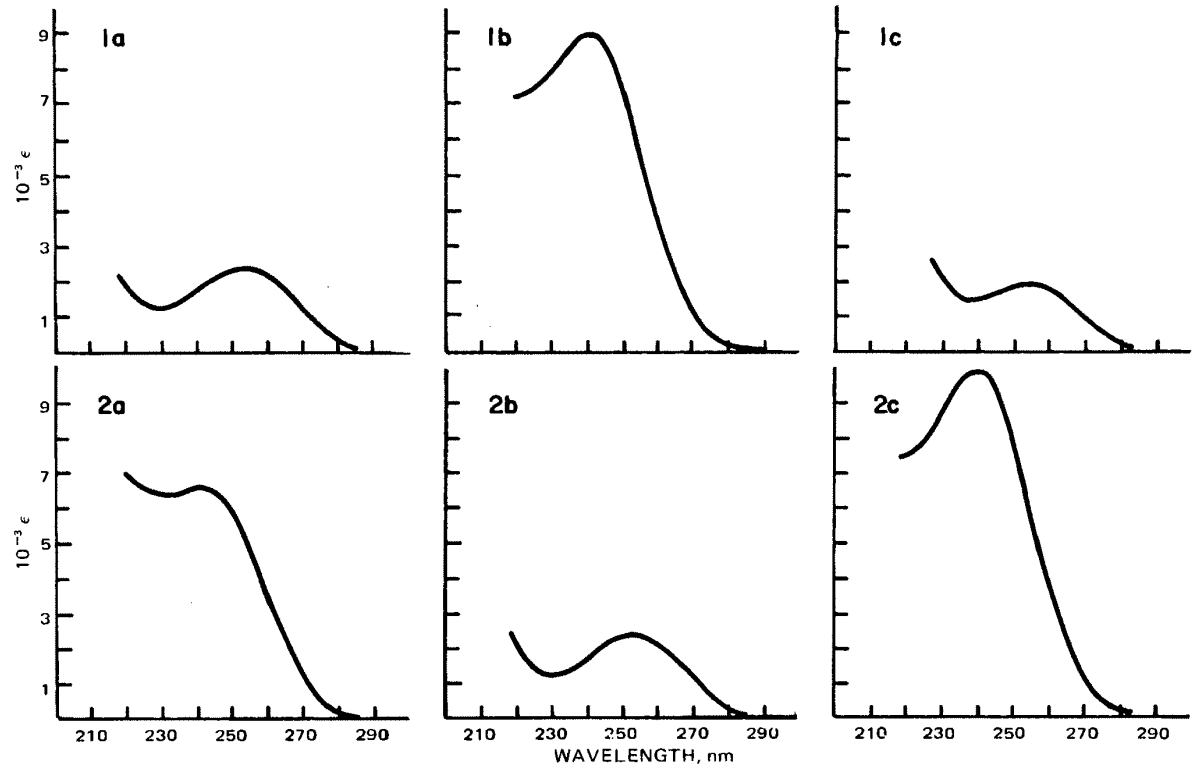


Figure 6—*Plots of apparent molar absorptivity* versus *wavelength for 5-azacytidine in solutions that were originally acid (1a), then adjusted to neutrality (1b), and reacidified (1c), or that were prepared at neutral pH (2a), were acidified (2b), and were reneutralized (2c).*

versus buffer concentration were extrapolated to zero buffer using least-squares linear regression analysis to obtain the values for these constants in the absence of buffer (Table III). In all cases, buffer plots of k_b were linear and showed a positive slope. But plots for k_a had positive slopes at pH 6.52 and 7.59, showed no buffer catalysis at pH 5.60 and 5.42, and indicated inhibition by the buffer at pH 4.62 (Fig. 5). No buffer sensitivity was seen for k_{obs} at pH 3.58 or 2.58.

Evidence for Addition across 5,6-Double Bond of 5-Azacytidine—If the UV spectrum of a 5-azacytidine solution is recorded as a function of pH, that spectrum is dependent on the order of the pH adjustment as well as on the pH itself (Fig. 6). For example, a neutral solution (2*a* in Fig. 6) has a spectrum that is less intense than that of a solution of equal concentration and pH that has been prepared from a previously acidic solution (1*b* in Fig. 6).

Clearly, acid at least partially converts the 5-azacytidine to a form that has a greater absorptivity at neutral pH than the 5-azacytidine itself. However, this rapid conversion does not occur at neutral pH since acidification of an originally neutral sample results in a solution whose spectrum is identical to that of an authentic sample in acid. Furthermore, if the neutral solution that was made acidic is readjusted to neutrality, the spectrum resembles one from an authentic sample in acid adjusted to neutrality (1*b* and 2*c* in Fig. 6).

The degree of difference between a neutralized solution (such as 1*b* in Fig. 6) and a neutral solution (2*a* in Fig. 6) depends upon the acidity of the initial solution. In Fig. 6 the initial acid concentration was 0.1 *N* HCl, and the apparent absorptivity at the absorption maximum for the solution adjusted to pH 6.5 was 9.03×10^3. When the initial acidity was 0.001 *N* HCl, the maximum absorptivity at pH 6.5 was 7.37×10^3. It was consistently observed that solutions made neutral and then reacidified showed decreased maximum absorptivities compared to the original acidic spectra as illustrated for the example in Fig. 6. These changes cannot be attributed to time or dilution factors. This decrease was not observed when solutions were adjusted from 0.1 to 0.001 *N* HCl and back to 0.1 *N*.

When experiments identical to those illustrated in Fig. 6 were carried out using 5-azacytosine, all spectra in 0.1 *N* HCl were identical, as were all spectra at pH 6.5.

Increasing concentrations of bisulfite brought about a progressive loss in intensity of spectra of both 5-azacytidine and 5-azacytosine. When spectra changed with time, initial absorbances were estimated by extrapolating plots of absorbance *versus* time to zero time. When these estimates were used, 5-azacytidine showed apparent molar absorptivities at 240 nm of 5.78×10^3, 3.60×10^3, and 1.38×10^3 in 0.001, 0.005, and 0.025 *M* bisulfite, respectively. Increasing the ionic strength by addition of sodium chloride did not alter the spectrum of the 0.001 *M* solution. In 0.001, 0.005, and 0.025 *M* bisulfite, 5-azacytosine had apparent molar absorptivities of 3.65×10^3, 1.55×10^3, and 0.37×10^3, respectively.

Spectrophotometric Determination of pKa Values for 5-Azacytosine—Absorbances at λ_{max} for the anionic form were plotted *versus* pH. Absorbances were corrected for dilution caused by the pH adjustment using:

$$A_{corr} = A_{exp}(V/V_0) \qquad \text{(Eq. 20)}$$

where V is the volume after addition of acid or base, and V_0 is the original volume. Figure 7 shows the plot obtained. The two pKa values were estimated from the midpoints of the curves and by use of Eqs. 21 and 22 for the pKa's of the conjugate acid and neutral molecule, respectively (8):

$$\text{pKa} = \text{pH} + \log[(A_I - A)/(A - A_N)] \qquad \text{(Eq. 21)}$$

$$\text{pKa} = \text{pH} + \log[(A - A_N)/(A_I - A)] \qquad \text{(Eq. 22)}$$

The absorbance of the solution when all the substrate is ionized is represented by A_I; A_N represents a solution of the neutral molecule; and A is the observed absorbance at the given pH. Two or three pH values within 0.5 pH unit of the pKa's were used. The average pKa values were 2.64 for the protonated molecule and 8.10 for the neutral species.

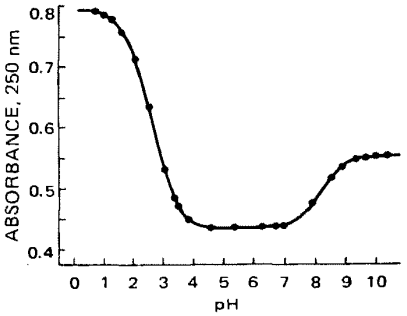


Figure 7—*Absorbance at 250 nm* versus *pH for* 1×10^{-4} M *5-azacytosine.*

DISCUSSION

Kinetics of 5-Azacytidine Hydrolysis in Hydrochloric Acid Solutions—Scheme II outlines the proposed path for 5-azacytidine loss in acid. The formation of 5-azacytosine and 5-azauracil was expected from previously reported results (5) and was confirmed by UV assay. TLC and spectral changes demonstrated that 5-azauracil was produced from the hydrolysis of 5-azacytosine. This was the expected source for the 5-azauracil in the nucleoside hydrolysis.

However, as may be seen in Fig. 2, the data indicate the immediate and rapid production of 5-azauracil. If it were produced solely from 5-azacytosine, a lag phase would be evident. Therefore, it was necessary to postulate the production of 5-azauracil by another, more rapid route. The most logical path involves formation of 5-azauridine as a reactive intermediate. It is proposed to have only a transitory existence and a low concentration and may be said to be in the steady state. This is supported by the absence of a lag time for the formation of 5-azauracil from 5-azacytidine. A lag phase would be expected if 5-azauridine had an appreciable lifetime. Moreover, there was no spectral evidence for the existence of 5-azauridine in the reaction samples.

The steady-state assumption requires that 5-azauridine be very unstable in the reaction system. This assumption is reasonable in view of what is known about the reactivity of the molecule. It is reported to cleave easily in hydrochloric acid at room temperature to form 5-azauracil and ribose. In dilute aqueous ammonia, 1β-D-ribofuranosylbiuret is formed. At pH 5 at room temperature, the UV spectrum of a solution disappeared in less than 1 hr (1).

The absence of 5-azauridine UV absorption was substantiated in the following manner. In neutral solution and as a solid, 5-azauridine actually exists in a form that exhibits no absorbance in the near UV (1). In ~0.1 *N* HCl, solutions have an apparent absorptivity of ~1000 at the λ_{max} of 241 nm (1, 9). Therefore, significant amounts in the reaction would be detected in the UV spectra of the reaction samples. This was tested by using the concentrations calculated for 5-azacytidine, 5-azacytosine, and 5-azauracil at various times during the reaction to generate spectra for the reaction mixtures. These calculated spectra were identical to the actual spectra in the region examined, 235–270 nm. This could not occur in the presence of an appreciable concentration of 5-azauridine unless its spectrum exactly matched that of one of the other components or their absorptivities differed by a constant factor at all wavelengths. Since 5-azacytosine, 5-azauracil, and 5-azacytidine have λ_{max} values of 247, 233, and 255 nm, while 5-azauridine is reported to have its λ_{max} at 241 nm, neither of these conditions can be met.

Since all of the 5-azacytidine loss could not be accounted for by the production of 5-azacytosine and 5-azauracil, it was necessary to include a third path for loss of the substrate to produce nonchromophoric products, probably resulting from fission of the triazine ring (5) (represented by k_{14} in Scheme II). While it is possible that 5-azauridine also contributes to the formation of nonchromophoric products, this pathway would be kinetically equivalent since 5-azauridine is in the steady state.

Data for 5-azacytosine hydrolysis were used to complete Scheme II. Rate constants shown in Scheme II were calculated at 70° (Table I). The observed first-order rate constant for loss of 5-azacytidine decreased at lower pH. This trend was seen also at 50°, as shown in Table II. As the acid concentration was increased, however, a larger fraction of 5-azacytidine formed 5-azacytosine and 5-azauracil (24% in 0.25 *N* HCl as opposed to 11% in 0.1 *N* HCl). 5-Azacytosine showed a similar pH dependency. Only 32% of the 5-azacytosine formed 5-azauracil in 0.1 *N* HCl, and 50% followed this route in 0.25 *N;* the overall rate constant for loss decreased in the more concentrated acid.

Therefore, it appears that increased acid concentration decreases ring opening reactions that produce nonchromophoric products, thereby forcing more substrate to undergo deamination and, in the case of 5-azacytidine, sugar loss. Since these latter two reactions are relatively insensitive to pH changes over the region examined, the overall rate of substrate loss decreased.

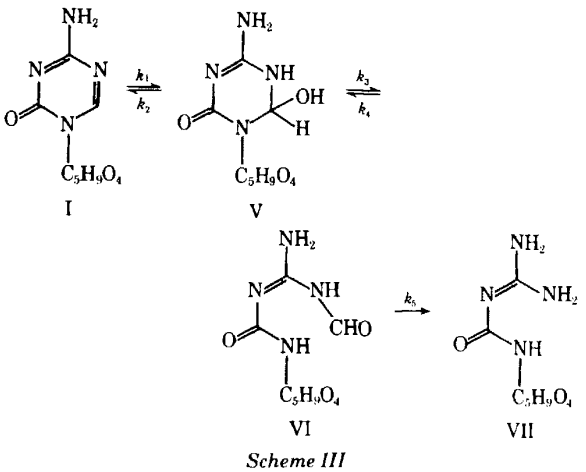
5-Azacytidine Hydrolysis in Presence of Buffers—In formic acid buffer, pH 2.6 and 50°, the loss of absorbance of a 5-azacytidine solution follows first-order kinetics, with the final absorbance being essentially zero. This same behavior is seen in hydrochloric acid solutions more dilute than 0.1 *N*. However, at pH 3.6 the absorbance plots of acidified samples are no longer linear. From pH 4.6 to 7.6, the absorbance plots are biphasic (showing a rapid initial drop in absorbance) and can be separated into two phases by the method of feathering (7) (Fig. 3). Reactions at pH 7.6 and 6.5, followed using nonacidified samples, were also biphasic. In this case the initial change was a rapid increase in absorbance, which was then followed by a slower decrease. An example is shown in Fig. 4. The apparent first-order rate constants obtained by feathering were the same whether acidified or nonacidified samples were used.

Several possible causes of this biphasic behavior were considered: (*a*) the presence of an impurity; (*b*) an A → B → C reaction where the initial phase represents the loss of A to B and the second phase represents the loss of B to C; and (*c*) a reaction with an initial reversible step (or steps) such as A ⇄ B → C (or A ⇄ X ⇄ B → C). The first possibility was eliminated by running a pair of reactions using 5-azacytidine purchased from two suppliers[4]. The absorbance *versus* time plots were identical, making an impurity an *unlikely cause for the nonlinear behavior.*

Samples taken during the second phase of the reactions, diluted in acid as shown in Table IV, and allowed to react at 70° showed rate constants that agreed with those found for 5-azacytidine. In addition, samples taken throughout the reaction and acidified had spectra that were essentially identical with that of 5-azacytidine at wavelengths longer than 240 nm. Therefore, it appears that 5-azacytidine is present throughout the reaction. This indicates that the formation of B must be reversible, thus eliminating the second reaction scheme.

A pathway with a reversible step is supported by the work of other investigators who reported the reversible formation of the *N*-formyl derivative of ribosylguanylurea (VI in Scheme III) in the decomposition of 5-azacytidine in buffer solutions (5).

By using results of previous work with 5-azacytidine and what is



Scheme III

[4] Schwarz/Mann and Aldrich.

known about the hydrolysis of imines, together with the experimental observations reported here, it is possible to develop a reaction scheme for 5-azacytidine in the presence of buffers as depicted in Scheme III.

Studies of imine hydrolysis (10) indicate that water attack to form a carbinolamine intermediate (V) is fast at low pH since attack proceeds by way of the protonated imine. Loss of free amine (V → VI) becomes the rapid step at higher pH values. This is consistent with the results of pH change experiments, which indicate that formation of an intermediate is rapid in acid but slow at neutral pH, while the reaction of this intermediate to form a third compound is rapid in buffer. This interpretation is also strengthened by the fact that more intermediate appeared to form at pH ~1 (0.1 *N* HCl) than at pH ~3 (0.001 *N* HCl). Based upon what is known about cytosine and its nucleosides (11), the pKa of 5-azacytidine should be little different from the 2.59 found for 5-azacytosine. Therefore, at pH 1, the sample, being nearly all in the protonated form, should contain more hydrate than the pH 3 sample, where more than half of the compound is not protonated.

If water adds across the 5,6-double bond, it seems reasonable that a better nucleophile, such as bisulfite, that is known to attack double bonds of heteroaromatic molecules (12, 13) would also add. Indeed, it was found that with increasing concentrations of bisulfite, the absorbance of 5-azacytidine solutions became progressively less intense, indicating an essentially nonchromophoric product as might be expected from an addition that brings about a loss of conjugation (14).

Although bisulfite adds to 5-azacytosine under the same conditions used for addition to 5-azacytidine, no evidence of hydrate formation was seen when 5-azacytosine was carried through the same pH adjustment experiments as the nucleoside. This finding suggests that the sugar group may be important in the hydration reaction. The 5′-hydroxy could act as a general base, making water a better nucleophile. It is especially well suited to act through a "concerted" mechanism, such as that proposed (15) for the addition of alcohols to phthalimidium cations.

The lack of significant hydrate formation with 5-azacytosine is also demonstrated by the spectral curve for pKa determination (Fig. 7). The protonated form of the molecule has a higher absorptivity than the neutral form. Conversely, 5-azacytidine absorbs less in acid than at neutral pH, presumably due to formation of the nonabsorbing intermediate. This loss of absorbance can be used to estimate the fraction of 5-azacytidine present in the hydrated form in acidic solution. Cytosine nucleosides and bases typically absorb more in the protonated than in the neutral form.

For example, the maximum absorbance of protonated forms of 6-methylcytidine and 6-methylarabinosylcytosine is about 1.7 and 1.6 times greater than that of the neutral molecules at the same wavelength. The protonated form of the base 6-methylcystosine absorbs 1.8 times as strongly as the uncharged form (11). At the wavelength of maximum absorption, protonated 5-azacytosine absorbs 1.6 times more than its neutral form. Without hydration, 5-azacytidine would be expected to exhibit the same pattern. Comparison of the observed absorptivity of 5-azacytidine in acid to that projected from the absorptivity of the unprotonated form (using the typical ratios just described) implies that roughly 70% of the 5-azacytidine exists as the hydrate in 0.1 *N* HCl.

Although the structure for Compound VI in Scheme III was proposed previously, it was neither successfully isolated from the reactions nor synthesized (5). Attempts at synthesizing compounds of this type by formylation of guanylurea led to recovery of either starting material or 5-azacytosine. Formation of 5-azacytosine in this way confirms the reversibility of the ring-opening step. The strong absorbance of analogous compounds such as *N*-formyl-*N*′-cyanoguanide was cited as evidence for formation of a compound like VI, since it could thereby account for the initial increase in absorbance observed in buffered samples (5). The fact that this increase occurs with no apparent lag phase emphasizes that loss of V to VI is rapid compared to formation of V from I.

Pithova *et al.* (5) identified ribosylguanylurea (VII) as a product of the hydrolysis of 5-azacytidine and showed that VII further decomposes to yield guanidine. In the present experiments, a λ_{max} of 221 nm at pH 7.6, observed in spectra for reaction mixtures, was attributed to the formation of VII. First-order plots using absorbance indicated that VII did not show appreciable absorbance at wavelengths longer than 240 nm in acid or buffer.

Table V—Results of Analog Computer Simulation of Scheme III

k_1	k_2	k_3	k_4	k_5	k_a[a]	k_b[a]	k_{obs}[a]
0.9	0.05	5.0	5.0	0.3	0.95	0.14	
					0.83[c]	0.14[c]	
0.7	0.1	7.0	7.0	0.1	0.70	0.044	
					0.72[c]	0.044[c]	
0.7	0.4	7.0	7.0	0.1	0.83	0.037	
					0.84[c]	0.036[c]	
					0.87[b]	0.038[b]	
0.7	0.7	7.0	7.0	0.1	1.01	0.031	
					0.93[c]	0.031[c]	
					1.03[b]	0.031[b]	
7.0	7.0	0.7	0.7	0.1	1.07	0.031	
1.0	7.0	0.7	0.7	0.1	0.86	0.010	
7.0	1.0	0.7	0.7	0.1	1.34	0.044	
7.0	7.0	0.5	0.5	0.5	1.10	0.105	
7.0	7.0	0.2	0.2	0.1	0.42	0.029	
7.0	7.0	0.2	0.2	0.5	0.72	0.066	
7.0	7.0	0.2	0.2	1.0			0.079
7.0	7.0	0.2	0.2	5.0			0.093
7.0	7.0	0.2	0.2	9.0			0.095

[a] All rate constants were obtained from semilog plots of I + V *versus* time unless otherwise noted. Apparent first-order rate constants are expressed as time^{-1}. [b] Obtained from data for I only. [c] Obtained from plots of 0.3 (I + V) + VI.

As stated under *Results*, the values of k_a and k_b obtained experimentally were the same using UV absorption data for the buffered reactions with or without acidification. It was of interest to determine whether Scheme III could provide data that would behave in this manner. Therefore, Scheme III was programmed using an analog computer. The values of the microconstants used are listed in Table V. Various components or combinations of components were studied as a function of time to simulate the spectral changes seen during the reactions.

According to Scheme III, I, V, and VI are present throughout the reaction. Using acidified samples, VI does not absorb at the wavelengths used for kinetic studies. When samples are acidified, I and V equilibrate so that the absorbance obtained actually reflects the sum of their concentrations in the reaction, although V has very little, if any, absorbance. In buffered samples, I and VI are responsible for the largest part of the absorbance, with VI absorbing more strongly than I.

With these considerations in mind, the analog program was adjusted to provide various kinds of data. The time course of I + V *versus* time was used to represent acidified samples. To simulate spectral changes seen with buffered samples, 0.3 (I + V) + VI was generated. The factor 0.3 was arbitrarily chosen to simulate the effect of differences in absorptivity. The time course of I alone was

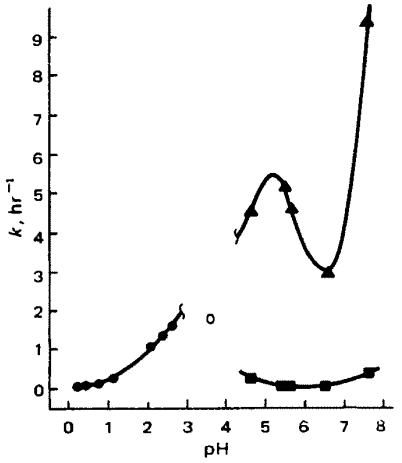
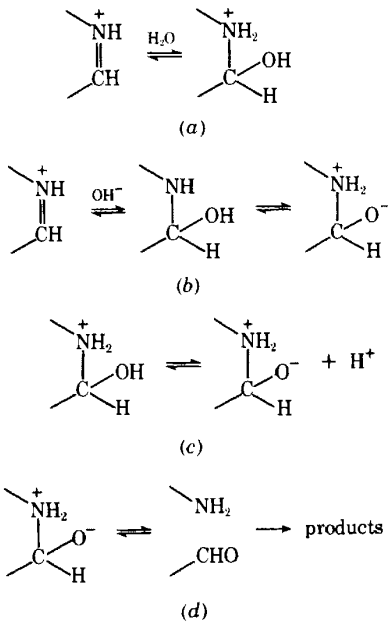


Figure 8—*Apparent first-order rate constants* k_a (▲), k_b (■), *and* k_{obs} (●) *for hydrolysis of 5-azacytidine at 50° as a function of pH. The point at pH 3.58 (○) was obtained as described in the text.*



Scheme IV

also defined. Plotting and feathering the data as described earlier gave estimates for k_a and k_b. These values were the same regardless of which simulation was used (Table V).

Hydrolysis of 5-Azacytidine as a Function of pH—Figure 8 shows a plot of observed first-order rate constants for 5-azacytidine hydrolysis as a function of pH. It is obvious from Scheme III that these are complex kinetic constants composed of several microconstants. This situation precludes an unequivocal interpretation of the pH–rate profile. However, Scheme III can be used in conjunction with present data to suggest mechanisms consistent with observed pH effects.

Both rate constants k_a and k_b are shown for the biphasic region (pH >4) in Fig. 8. Based on Scheme III, k_b may be defined as:

$$k_b = fk_5 \qquad \text{(Eq. 23)}$$

where f is the fraction of (I + V + VI) present as VI. The values for k_5 cannot be calculated from the observed constant k_b, since the value for f is not known. However, it is apparent from Fig. 8 that the product fk_5 is relatively independent of pH. Conversely, k_a is pH dependent. Although it is recognized that k_5 can affect the value of k_a, the observed pH dependency is most likely due to changes in the relative rates of the reversible steps for hydrolysis of I to VI. In the 4.5–6.5 pH region, hydrolysis of the triazine ring may be visualized as a series of steps analogous to those postulated for imine hydrolysis (Scheme IV).

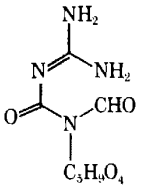
As in imine hydrolysis (10, 16), formation of protonated carbinolamine or neutral carbinolamine (Scheme IV, *a* and *b*) *via* the reactive cationic substrate (IH^+) would be the slowest reversible step at pH 6.5. As the pH decreases, the concentration of IH^+ increases; therefore, the apparent first-order rate constant increases. This increase would be expected to continue until protonation of I was complete, but increasing acidity inhibits formation of the zwitterion (Scheme IV, *c*) so less of it is available to react. Thus, near pH 5 the observed rate constant begins to decrease.

As the pH decreases further, first-order plots become linear instead of biphasic. Although the rate of formation of protonated V (VH^+) from IH^+ becomes faster, the fraction in the reactive form (zwitterion) decreases. The apparent first-order rate constant for loss of IH^+ is now attributed to cleavage of the carbinolamine (Scheme IV, *d*) as described by Scheme V.

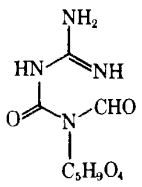
$$IH^+ \underset{\text{fast}}{\rightleftharpoons} VH^+ \underset{\text{slow}}{\rightleftharpoons} VI \underset{\text{fast}}{\longrightarrow} VII$$

Scheme V

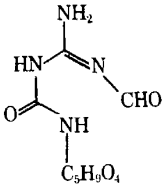
Since the hydration of substrate to form VH^+ is fast, the overall loss appears to be simple first order. The observed constant continues to decrease with increasing acidity due to the decreasing fraction of reactive species in the form of zwitterion. Eventually, loss of IH^+ by this path becomes sufficiently slow that formation of 5-azacytosine and 5-azauracil becomes important at a pH of about 1, but k_{obs} continues to decrease in this region, presumably *via* the reduction of zwitterion.



VIII



IX



X

The observed increase in k_a at pH 7.6 may result from a pathway involving base-catalyzed water attack on the neutral molecule as observed for certain imines (10).

Scheme IV shows the result of 5,6-addition of water. Cleavage of the ring would then result in Compound VIII. Addition across the 1,6-bond would be necessary to obtain Structure VI, which was suggested previously (5). Such addition appears possible starting from the resonance form of IH^+, which has the positive charge at C-6. Addition of water could then occur across the 5,6- or the 1,6-bond. Since a positive identification of the compound was not made (5) and both VI and VIII yield the same product upon hydrolysis of the amide, both routes are possible. Initial protonation could also occur at the 3-nitrogen, leading to Compounds IX and X, which differ from VI and VIII only in the position of a proton.

Implications for Stability Prediction—This report is the first one to suggest the formation of the proposed 5,6-hydrate of 5-azacytidine. While the hydrate was not isolated, considerable indirect evidence has been presented in support of its existence. Regardless of the exact structure for the proposed intermediate, its facile formation in acidic solutions of 5-azacytidine is readily apparent. The undetermined biological significance of this previously unreported intermediate, along with the complexity of the observed first-order rate constants (as illustrated by the analog simulations), indicates the difficulty in predicting stability of 5-azacytidine potency in solutions.

For example, although the apparent rate constants for loss in acidic solutions are less than the k_a values obtained in buffers, a large fraction (about 0.7) of the drug forms the proposed hydrate immediately upon dissolution in acid. The rate constant observed in acid applies only to the loss of drug after the initial equilibration between drug and hydrate has taken place. Therefore, if the hydrate is not itself biologically active or is not bioreversible to drug, most potency is lost long before predictions based on the observed rate constant would have indicated significant hydrolysis.

Equation 19 describes the absorbance changes for 5-azacytidine in buffer solutions. The rate constant values are the same with or without acidification, but the values of the coefficients A and B differ. Provided that Scheme III applies, it is assumed that neither VI nor VII absorbs in acid at the wavelengths used in this study. It is also assumed that the proposed hydrate (V) does not accumulate to a significant extent at neutral pH. Although these assumptions were not proven, they were justified in previous discussion. By using these assumptions, the absorbance of acidified samples of reactions run in the buffer region may be attributed to 5-azacytidine and Eq. 19 may be written:

$$\text{percent remaining} = 100\,(Ae^{-k_a t} + Be^{-k_b t}) \qquad \text{(Eq. 24)}$$

Table VI—Times to Reach 90, 80, and 70% of Original Concentration of 5-Azacytidine in Buffer Solutions at 50° (Predicted using Eq. 24)

pH	Total Buffer[a]	Minutes 90%	80%	70%
	Phosphate			
7.59	0.615	2.5	7	14
	0.033	3.5	10	22
6.52	0.20	6	15	32
	0.04	11	32	81
	(0.04	80	225)[b]	
5.42	0.33	3.5	9	17
	0.066	6	18	52
	Acetate			
5.60	0.44	6	15	34
	0.088	6.5	18	56
4.62	0.80	4	10	16
	0.16	5	11	20

[a] Adjusted to $\mu = 0.5$ with sodium chloride (Table III). [b] At 30°.

This equation may be used to calculate the percent drug remaining at any time by employing data for acidified samples, provided the above conditions are met. Failure of the first condition would lead to inapplicability of the biexponential equation. If the second assumption does not hold, the actual percent remaining will be less than that calculated from Eq. 24.

The percent drug remaining can be calculated using the appropriate constants in Eq. 24, keeping in mind the assumptions involved. For example, the values for A, k_a, B, and k_b listed in Table III were used to estimate the time required to lose 10, 20, and 30% in a variety of buffers at 50°. Results are shown in Table VI, where it can be observed that each succeeding 10% loss requires a longer time. This result is due to the transition from the rapid initial phase to the slower terminal phase. It is apparent that 5-azacytidine is decidedly unstable under these conditions. The maximum time required to lose 10% of the drug is 11 min, and 20% is lost in 32 min under these conditions.

To estimate the loss of potency under potential clinical conditions, a reaction was studied at 30° at the pH of maximum stability observed at 50° (0.02 M Na_2HPO_4–0.02 M NaH_2PO_4–0.42 M NaCl, pH 6.5). Within 30 min, 5% of the drug was lost; after 80 min, 10% was lost; and 20% was gone in 225 min. This apparent instability of 5-azacytidine suggests that it would be prudent to prepare solutions shortly before use to avoid significant degradation. However, it requires more than 2 hr for potency to decrease from 90 to 80% and the decrease from 80 to 70% would take even longer. It is likely that solutions used within 6 hr contain at least 70% of the original drug.

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Phase II Study of 5-Azacytidine in Solid Tumors [1,2,3,4]

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SUMMARY

A phase II study utilizing 5-azacytidine in the treatment of patients with solid tumors was carried out by the Southwest Oncology Group (SWOG-7208). Of 214 patients entered in the study 191 were eligible and 167 were evaluable. While initially they received 225 mg/m² iv on Days 1–5 every 3 weeks, because of toxicity the dose was subsequently reduced to 175 mg/m² and later to 150 mg/m². Five partial regressions, 2.6% of the eligible patients and 3% of the evaluable patients, lasting from 28 to 77 days were observed. Sixteen patients, 8.4% of the eligible patients and 9.6% of the evaluable patients, had no significant change in their disease for 39–255 days. The major toxicities were myelosuppressive and gastrointestinal with 13 deaths attributable to drug toxicity: 11 due to sepsis and two due to cerebral hemorrhage. 5-Azacytidine induced few favorable responses; those that did occur usually were of poor quality and short duration and were associated with significant toxicity.

[Cancer Treat Rep 61:51–54, 1977]

5-Azacytidine is a pyrimidine analog of cytidine which was synthesized by Piskala and Sorm in 1964 (1). The drug acts as an analog of cytidine, interfering with the synthesis of DNA, RNA, and proteins. The mechanism of action has been investigated by a number of investigators, an extensive bibliography of which is included in a recent review by Carter and Slavik (2).

The drug exhibits experimental antitumor activity against L1210 leukemia in mice, lymphoid leukemia in AK mice, and Ehrlich ascites tumor in mice (3).[14] In a phase I study by Weiss et al (4) 30 patients with a variety of solid tumors were treated, with objective responses being reported in seven of 11 patients with carcinoma of the breast, two of five with malignant melanoma, and two of six with carcinoma of the colon. McCredie et al[15] noted a response in one patient with a renal carcinoma and in another with malignant melanoma.

In August 1972, the Southwest Oncology Group (SWOG) initiated a phase II study (SWOG-7208) in

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[2]Supported by Public Health Service grants CA-03389, CA-04915, CA-14028, CA-10379, CA-16385, CA-13238, CA-03096, CA-12014, and CA-16943 from the National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[3]This study was conducted by the Southwest Oncology Group (SWOG).

[4]5-Azacytidine: NSC-102816; CAS reg. No. 320-67-2; 5-triazin-2(1*H*)-one, 4-amino-1-β-D-ribofuranosyl-.

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[8]Department of Developmental Therapeutics, M. D. Anderson Hospital and Tumor Institute, Houston, Tex.

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[10]University of Utah Medical Center, Salt Lake City.

[11]Borgess Medical Center, Kalamazoo, Mich.

[12]Hematology-Oncology, Department of Medicine, University of Texas Medical Branch, Galveston.

[13]The following institutions also participated in this study: University of Kansas, Brooke Army Medical Center, Tulane University, Cleveland Clinic, Baylor College of Medicine, Ohio State University, Northwestern University, University of Oklahoma, Scott and White Clinic, Wilford Hall Medical Center, Louisiana State University, University of Virginia, Tumor Institute of Seattle, and University of Arizona.

**Reprint requests to:* SWOG, Operations Office, 3500 Rainbow Blvd, Suite 201, Kansas City, Kan 66103.

[14]Broder LE, and Carter SK. Clinical brochure: 5-azacytidine (NSC-102816). Prepared by the NCI, Bethesda, Md, 1970, 25 pp.

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order to determine the effectiveness of 5-azacytidine in patients with solid tumors.

METHODS AND MATERIALS

Patients with histologically proven malignant solid tumors with measurable disease who were not eligible for higher priority protocols or more conventional chemotherapy were eligible for this study. Other criteria for eligibility included the following: written consent by the patient who had been informed as to the investigational nature of the study; a life expectancy > 8 weeks; no prior therapy with 5-azacytidine; an adequate bone marrow reserve with an absolute granulocyte count > 2000/μl and a platelet count > 100,000/μl; adequate renal and hepatic function with a BUN < 25 mg/100 ml or serum creatinine < 2 mg/100 ml and total serum bilirubin < 2 mg/100 ml unless caused by the tumor; and no chemotherapy given in the previous 21 days. Pretreatment studies included a cbc count, blood chemistries, and chest and other roentgenograms as clinically indicated. Blood counts were obtained weekly, while chemistries and roentgenograms were obtained every 3 weeks.

The initial dose of 5-azacytidine was 225 mg/m^2 by rapid iv push or infusion over 15–30 minutes on Days 1–5 every 3 weeks as tolerated. Because of myelosuppression, the dose was subsequently reduced to 175 and later to 150 mg/m^2. Subsequent courses were repeated at 3-week intervals if the nadir of the wbc and platelet counts had been reached and evidence for recovery was present. The doses in subsequent courses were adjusted according to the prior nadirs.

Two courses of treatment associated with some myelosuppression were considered necessary for an adequate trial.

The patient and his performance status, as well as the above studies, were evaluated at a minimum of every 3 weeks. Hematologic toxicity was graded according to severity.

TABLE 1.—5-Azacytidine—phase II study of solid tumors (SWOG-7208)

	No. of patients
Eligible	191
Evaluable	121
Partially evaluable	46
Early death	32
Refused further therapy	6
Lost to followup	8
Not evaluable	24
Insufficient data	16
Other	8

The response to therapy was classified as: complete regression, partial regression, stable disease, and increasing disease. Standard criteria were used with a partial regression indicating a reduction in tumor by > 50%.

RESULTS

Tumor Response

One hundred and ninety-one eligible patients from 21 institutions were entered in this study (table 1). All patients had received prior therapy. The tumor types and the response to therapy are listed in table 2.

There were five partial regressions noted for a response rate of 2.6% of the 191 eligible patients and 3% of the 167 evaluable patients. Stable disease was noted in 16 patients for a response rate of 8.4% of the eligible patients and 9.6% of the evaluable patients.

Partial regressions were noted in two patients with adenocarcinomas of the lung, one with a squamous cell carcinoma of the lung, and two with embryonal carcinomas of the testicle. The duration of the responses varied from 28 to 77 days. Three of the five patients received 225 mg/m^2 while two received 175 mg/m^2. Stable disease was noted in five of 17 patients with adenocarcinomas of the kidney, one of 14 with adenocarcinoma of the breast, two of 15 with adenocarcinomas of the colon, two of six with adenocarcinomas of the pancreas, and in single patients with malignancies arising from six other primary sites. Responses were noted at all three dose levels with the duration of the stability varying from 39 to 255 days.

Toxicity

The major toxicities associated with 5-azacytidine therapy were myelosuppressive and gastrointestinal. At the higher dose leukopenia with a wbc count < 4000/μl was noted in 83% of the patients, with severe leukopenia (a wbc count < 2000/μl) being noted in 64%. At the lower doses 63% of the patients had a wbc count < 4000/μl while in 27% it was < 2000/μl.

At the higher dose 29% of the patients developed thrombocytopenia with 8% having a platelet count < 50,000/μl. At the lower doses 12% of the patients had thrombocytopenia which was severe in 4%.

The gastrointestinal toxicity did not appear to be dose related because at all dose levels > 80% of the patients had nausea and vomiting and/or diarrhea which was severe in 33%. The nausea and vomiting was usually most severe on the first and second

TABLE 2.—Tumor types and response to therapy

Tumor	No. of evaluable patients	No. of patients with— Partial regression	Stable disease	Increasing disease
Pancreas				
Adenocarcinoma	6		2	4
Islet cell carcinoma	1		1	
Lung				
Adenocarcinoma	12	2	1	9
Squamous cell carcinoma	14	1		13
Undifferentiated cell carcinoma	12			12
Colorectal	15		2	13
Kidney	17		5	12
Breast	14		1	13
Testicle				
Embryonal cell carcinoma	2	2		
Teratocarcinoma	1			1
Choriocarcinoma	1			1
Urinary bladder	2		1	1
Primary liver carcinoma	4		1	3
Palate, adenocarcinoma	1		1	
Parotid, adenocarcinoma	3		1	2
Uterus, adenocarcinoma	1			1
Cervix, squamous cell carcinoma	4			4
Ovary	5			5
Non-Hodgkin's lymphoma	4			4
Sarcoma	7			7
Melanoma	13			13
Tongue, squamous cell carcinoma	2			2
Nasal antrum	1			1
Esophagus, squamous cell carcinoma	3			3
Stomach, adenocarcinoma	7			7
Skin, squamous cell carcinoma	1			1
Larynx, squamous cell carcinoma	1			1
Gallbladder	1			1
Unknown primary				
Adenocarcinoma	8			8
Undifferentiated cell carcinoma	4			4
Totals	167	5	16	146

days but in some patients it persisted through the entire 5-day period of administration or longer. Antiemetics, even when given in large doses, were not noticeably effective. Diarrhea often occurred 2–3 hours following drug administration and usually remitted spontaneously. Twenty of the 191 eligible patients (11%) refused further therapy mainly because of the severe gastrointestinal toxicity. Hepatotoxicity was not observed (5).

In this study there were 13 deaths attributable to drug toxicity. Eleven were due to sepsis associated with severe neutropenia while two were due to cerebral hemorrhage associated with severe thrombocytopenia. Eleven deaths occurred on the higher dose schedule (225 mg/m^2) while two occurred at the lower doses (175 and 150 mg/m^2). Eight deaths occurred during the first course of therapy with death occurring in 11 of the 13 patients within 36 days. All patients had had previous treatment with radiation therapy, chemotherapy, or both, or had bone marrow infiltration with tumor rendering them more susceptible to the myelosuppressive toxicity.

DISCUSSION

Despite the early encouraging results of the study reported by Weiss et al and the Central Oncology Group (4), the results of this study were quite disappointing. 5-Azacytidine induced a few favorable responses which were usually of poor quality and short duration. The therapy was associated with significant toxicity with a death rate of 6% of all patients entered and with 11% of the eligible patients refusing further therapy. Moertel et al (6) had a similar experience in a phase II study of 5-azacytidine in the treatment of patients with advanced gastrointestinal cancer, reporting only one transient partial response out of 29 patients treated. They also reported significant toxicity. On the other hand, it should be noted that encouraging favorable

responses to this drug have been reported in the treatment of patients with acute leukemia, both myeloblastic and lymphoblastic (7–9).

Further clinical trials may be indicated in certain solid tumor types such as embryonal carcinoma of the testicle and adenocarcinomas of the lung and kidney. Caution is advised in patients with inadequate bone marrow reserves.

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News

Big interest in heavy drugs

The drug industry is seeking profits by modifying hydrogen in existing medications.

Katharine Sanderson

Substituting atoms in drugs could allow patenting opportunities and create better pharmaceuticals.

Bloomberg News/Landov/Photoshot

Pharmaceutical companies are beginning to bet on the idea that simply switching a hydrogen atom with a heavier isotope in a currently approved drug could create a better drug. Encouraged by results from clinical trials, companies are snapping up intellectual-property rights on many of the modified drugs.

On 16 March, for example, Concert Pharmaceuticals of Lexington, Massachusetts, reported results of a phase I clinical trial for a version of the antidepressant paroxetine, sold as Seroxat by GlaxoSmithKline and first marketed under that trade name in 1992. Concert's version swaps out one or more of the hydrogen atoms in the paroxetine structure (see **'Drug modifications'**) for deuterium, a heavier isotope of hydrogen that contains a proton and a neutron, rather than just a proton. The company was testing this deuterated version for treating hot flushes without the side effect of standard paroxetine in which the liver enzyme CYP2D6 is inactivated. Because this enzyme metabolizes many other drugs, inactivating it means it is hard to take other drugs along with paroxetine.

In Concert's trial, 94 women tested paroxetine — both standard and deuterated versions — along with the cough medicine dextromethorphan, which is metabolized by CYP2D6. Taken with standard paroxetine, metabolic uptake of the cough medicine is inhibited, says Roger Tung, chief executive at Concert. However, the deuterated version showed less metabolic inhibition — suggesting that the drug might be better to combine with others.

In theory, deuterated drugs can work differently in the body because deuterium can make stronger chemical bonds than hydrogen. This can affect how quickly a drug is broken down.

Another company working with deuteration is Auspex Pharmaceuticals, based in Vista, California. Last October, Auspex announced phase I clinical trial results with a deuterated version of venlafaxine. Venlafaxine was first produced by Wyeth, in 1993, as the antidepressant Effexor. The trial suggested that the deuterated version of the drug stayed in the bloodstream longer than the non-deuterated version and may even cause fewer side effects, says Mike Grey, chief executive of Auspex.

The company already has a patent on its deuterated version of venlafaxine, and has filed more than

150 other US patents on families of deuterated drugs. Concert has filed more than 100 US patent applications and has received two notices of allowance already, and is expecting to get its first patents this year.

Beyond the obvious

Kevin Mooney, a patent lawyer with Simmons & Simmons in London, says the strategy of piling up patent applications on deuterated versions of existing drugs is legitimate. "Everyone is entitled to research on other compounds," he says. Still, the onus will be on companies to demonstrate the usual criteria for patentability. "They would have to show that this deuterated form of paroxetine was new," he says, "then they would have to show that it wasn't an obvious thing to do." Patent applications — for example, those involving different salts of known compounds — often fail on this second requirement of non-obviousness.

Tung is confident that his company's tactics will work. "We treat this as an entirely new chemical entity," he says.

But in future, getting such patents will be harder, says Kirk Gallagher, a pharmaceutical patent lawyer at D Young and Co in London. "The obviousness bar will be raised as time goes by," he says, as the idea of deuterating a drug to get different pharmacokinetic properties becomes commonplace. Rather than the broad patents covering families of drugs being applied for now, companies will have to show improved action of specific molecules to convince a patent-giver that they have something new, Gallagher says. "They'll have to do more science before they file their patents."

Derek Lowe, a drug-discovery scientist and author of the In the Pipeline blog, says larger drug companies may take the same approach. "I think that every big pharma company is keeping this in mind," he says. "That's where the start-ups are going to have a problem."

The approach with deuterated drugs is similar to that used by Sepracor, based in Marlborough, Massachusetts, in the 1980s. Until that point, patents didn't include specific information on isomeric forms of drugs. Sepracor made its business by filing patents on active isomers of known drugs. In response, pharmaceutical companies began to routinely specify chiral isomers on patents. "Pharma companies will now probably add deuterium analogues," says Gallagher.

Grey isn't worried. "There is still a vast landscape for us to explore," he says. Auspex hopes to partner with larger drug companies to continue developing its products.

Concert is similarly looking for ways forward. The company has raised US$96 million in start-up money so far, but has decided not to develop its deuterated paroxetine further for now — focusing

instead on another deuterated drug, an HIV protease inhibitor, which it thinks will be a bigger seller.

A Phase I Study of 5-Azacytidine (NSC-102816)

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5-Azacytidine was first synthesized by Piskala and Sorm[1] in 1964, and their studies demonstrated that the drug was readily incorporated into both DNA and RNA. Its antineoplastic activity is attributed to its ability to inhibit DNA, RNA, and protein synthesis. This inhibitory action appears to be cell-cycle specific, and the greatest sensitivity is upon cells in the S-phase. Antitumor effect was demonstrated against L-1210 mouse leukemia with intermittent or continuous administration.

Studies with radioactive-labeled drug[3] indicated that absorption from subcutaneous injection sites was rapid, with peak plasma levels of radioactivity approximately equal to that noted in patients receiving the drug intravenously. The half-life after intravenous injection was 3.5 hours and after subcutaneous administration, 4.2 hours. Urinary excretion, however, was greater in patients receiving intravenous injections. The highest uptake of 5-azacytidine in tumor tissue was achieved when the drug was given intravenously. Small amounts of radioactivity were detected in the spinal fluid.

Phase I studies by Weiss and associates[2] and by Karon[4] indicated that the toxicity associated with the administration of this drug to humans was primarily nausea, vomiting, and diarrhea. These studies further indicated that these troublesome side effects appeared to be related to the amount of drug given in each injection. Hematologic toxicity consisted of granulocytopenia and thrombocytopenia usually occurring within three weeks after the start of chemotherapy. Transient elevations of SGOT were seen in some patients but there was no other evidence of hepatic toxicity.[5]

Weiss noted clinical responses in 7 of 11 patients with carcinoma of the breast, 2 of 5 patients with melanoma, and 2 of 6 patients with carcinoma of the colon. Subsequent studies with this drug in 29 patients with advanced gastrointestinal carcinoma revealed the anticipated toxicity with only one transient and partial objective response.[6] Tan and associates[7] studied 21 patients, 9 of whom were children, using intravenous 5-azacytidine. Leukopenia and thrombocytopenia occurred in all patients; nausea and vomiting was often severe but diminished with divided doses of the drug. Occasional ab-

From the Eastern Cooperative Oncology Group (ECOG) (Paul P. Carbone, Chairman) Supported in part by grants CA-02824 and CA-02822 from the National Cancer Institute, National Institutes of Health. 5-Azacytidine was supplied by Cancer Therapy Evaluation, DCT, NCI. 5-Azacytidine NSC 102816; CAS reg. no. 320-67-2; s-triazin-2(1H)-one-4-amino-1β-D-ribofuranosyl.

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dominal pain, diarrhea, and fever were also noted. No regressions were seen in the patients with solid tumors of lymphomas in this series. One of 7 acute lymphatic leukemias had a poor remission. Karon et al.[4] studying 34 children with advanced acute leukemia receiving doses of 5-azacytidine ranging from 2 to 300 mg/m^2 daily X 5 noted responses in 6 of 12 myelocytic leukemias and 4 of 22 lymphocytic leukemia. Cunningham et al.,[8] using the agent in the treatment of carcinoma of the breast and comparing it with CCNU, noted a low response rate for both agents (two of 19 patients treated with CCNU and 2 of 21 patients treated with 5-azacytidine). Vogler et al.,[5] reporting on the phase I studies of the Southeastern Cancer Study Group, noted that 5-azacytidine given twice weekly at doses of 150–200 mg/m^2 would produce granulocytopenia after several weeks associated with nausea, vomiting, and diarrhea. No renal or hepatic toxicity was noted. Bellet et al.,[9] using the subcutaneous route of administration, noted only mild gastrointestinal toxicity at doses producing significant bone marrow depression. Hepatic toxicity occurred in five patients, possibly related to extensive hepatic involvement with metastatic tumor.

This report concerns the preliminary phase I study done by the Eastern Cooperative Oncology Group with this agent in patients with solid tumors.

Our study was undertaken as an attempt to determine the tolerated dose for the daily and once-weekly schedules of administration in the event that more definitive therapeutic studies were to be undertaken by the group.

Methods and Materials

Patients were selected for study who had progressive disease and who were not considered candidates for other group studies with a higher priority and for whom no other therapy was available. All had to be in reasonably good status and to have a life expectancy of at least three months. They were to have no evidence of bone marrow suppression and were to have been off all prior myelosuppressive or hormonal therapy and x-ray therapy for at least six weeks prior to entering the study. Cases were entered in a sequential pattern with only one patient at each of the cooperating institutions entering study per week for each schedule of drug administration. Four patients were to be entered at each level for each dose schedule during the initial phase of the study before proceeding to the next dose in the escalation pattern.

Participants could be withdrawn from the study at any time either upon request of the patient or the investigator if it was deemed in the best interest of the patient. No patient was to reenter the study after completing an initial drug trial unless eight weeks had elapsed from the time of the last drug dose.

Participants in the study were randomly assigned to either of the two groups. The first group received 5-azacytidine once weekly intravenously for a period of four weeks, and the second group received 5-azacytidine daily for five days followed by a nine-day observation period. A second course of five days was given after the observation period. At the end of four weeks, both treatment groups were observed for an additional four-week period for delayed toxicity or to allow toxicity to regress before being removed from the study.

The initial starting dose for the weekly schedule was 200 mg/m^2, and the starting dose for the five-day daily injection schedule was 50 mg/m^2. Incremental increases of both drugs were taken in a stepwise fashion as follows: step (1), increase by 100 per cent of starting dose; step (2), increase by 66⅔ per cent of starting dose; step (3), increase by 50 per cent of starting dose; step (4), increase by 33⅓ per

cent of the starting dose; and step (5), increase by 25 per cent of the starting dose. Weekly evaluations were made of drug toxicity and progress of the study by telephone conference calls among the participating investigators. No incremental increase or assignment of new patients to a new dose level was made without prior evaluation of the status of all patients actively participating in the drug study. Serial laboratory tests were performed according to the protocol design, and x-rays and measurements of lesions were made at two-week intervals where appropriate. The study was to be terminated when a tolerated dose without prohibitive toxic side effects was established for both the daily and weekly schedules of drug administration.

5-Azacytidine was reconstituted with 5 to 10 ml sterile water and administered by rapid intravenous push. After the first patients developed severe nausea and vomiting, chlorperazine (Compazine) was given parentally 15 minutes prior to the intravenous administration of 5-azacytidine. Chlorperazine by suppository was repeated during the next 24–48 hours as required.

Results

Twelve patients received 21 courses of 5-azacytidine by the daily schedule and 15 patients received 50 weekly courses of drug by the weekly schedule (Table I). Table II shows the toxicity encountered at each of the dose levels for the daily schedule. Nausea and vomiting were the most prominent toxic side effects and occurred in every patient. Phenothiazines were only partially successful in relieving these symptoms. The vomiting usually had its onset 30 minutes to 2½ hours after the injection of 5-azacytidine and in some instances persisted for as long as 12–16 hours. In some patients, this was associated with diarrhea. As the severity of the nausea and vomiting increased, so did the degree of weight loss, and some patients refused to continue in the study after having severe nausea and vomiting during the first course of therapy.

TABLE I

Patient Distribution of Dose and Schelule of 5-Azacytidine

Dose (mg/m²)	No. of patients	No. of courses
	Daily Schedule	
50	4	7
100	3	6
133	3	5
158	2	3
Total	12	21
	Weekly Schedule	
200	4	14
400	4	15
533	5	15
633	2	5
Total	15	50

Aside from the gastrointestinal toxicity (Tables II and III), we encountered no stomatitis and only one episode of skin rash. This was a transient mild maculopapular eruption which occurred during the fourth week of the weekly schedule of drug administration at a dose of 200 mg/m². One patient on the daily schedule and one patient on the weekly schedule showed a rise in blood urea nitrogen which returned to normal when the drug was discontinued. Two patients on the daily schedule also had a rise in uric acid from normal values prior to therapy to 9.6 and 10 mg/100 ml after completion of a five-day course of drug. (One of these patients also had both an increase in blood urea nitrogen and uric acid.) One possible instance of hepatotoxicity occurred in a patient receiving drug weekly as evidenced by a rise in SGOT from 30 units to

TABLE II

Daily Schedule Toxicity*

Patient	Carcinoma	Total 5-azacytidine dose (mg)	Wt. loss (kg)	Nausea	Vomiting	Diarrhea	Hematologic	Comments
				50 mg/m²				
O.S.	Endometrium	325	2	3	3	0	0	refused second 5-day course
P.M.	Lung	1200	0	2	2	0	0	
				1	1	0	2	Compazine
E.R.	Colon	720	0	2	2	1	0	
				1	1	1	0	Compazine
O.G.	CNS	700	0	1	1	0	0	
				0	0	0	2	Compazine
				100 mg/m²				
R.L.	Leiomyosarcoma	1500	1	2	2	2	0	
				2	2	2	0	
E.L.	Cervix	825	0	1	2	0	0	
				1	1	0	0	
J.G.	Lung	1500	2	2	2	0	0	
				0	0	0	0	Compazine
				133 mg/m²				
L.A.	Unknown	1575	3	1	2	0	0	
				1	1	1	0	Compazine
K.B.	Prostate	1000	1	2	2	0	4	rise in BUN (17-41 mg%)
J.M.	Colon	1125	3	2	1	0	1	rise in BUN (12-38 mg%)
				2	1	0	2	uric acid up to 9 mg%
				158 mg/m²				
W.S.	Lung	2000	2	2	2	1	3	
				2	2	2	4	
C.B.	Lung	2000	3	3	3	1	3	uric acid up to 10 mg%
				NO SECOND COURSE				

* Scale: 0, none; 1, mild; 2, moderate; 3, severe; 4, life threatening.

C.B.	Lung	2000	3	2	2	2	4	3	uric acid up to 10 mg%
				3	3	1			
				NO SECOND COURSE					

* Scale: 0, none; 1, mild; 2, moderate; 3, severe; 4, life threatening.

TABLE III

Weekly Schedule Toxicity*

Patient	Carcinoma	Total 5-azacytidine dose (mg)	Wt. loss (kg)	Nausea	Vomiting	Diarrhea	Hematologic	Comments
				200 mg/m²				
C.M.	Lymphosarcoma	1499	1	2	2	2	0	no control by Compazine, skin rash
P.M.	Larynx	1280	1	0	3	2	1	V & D after 4th inj.
J.M.	Melanoma	720	0	1	1	1	0	
B.D.	Melanoma	1400	0	1	1	1	0	
				400 mg/m²				
M.H.	Breast	1800	1	2	2	0	0	refused drug after 3rd dose
C.H.	CNS	1400	1	2	2	3	4	hematologic
M.R.	Prostate	2800	0	1	1	1	0	Compazine
M.J.	Tongue	2360	1	1	1	1	0	Compazine
				533 mg/m²				
J.C.	Stomach	800	1	3	3	2	0	refused to continue
J.C.	Cervix	1600	1	3	3	0	0	refused after 2nd injection
J.L.	Esophagus	3000	2	2	1	1	0	Compazine
J.A.	Unknown	3775	3	2	2	1	1	Compazine
C.B.	Lung	3100	2	2	2	1	1	Compazine
				633 mg/m²				
L.C.	Colon	3228	3	3	3	3	1	BUN rise (10-26 mg%)
R.B.	Kidney	3420	3	3	3	3	2	SGOT rise from 30-72 units

* Scale: 0, none; 1, mild; 2, moderate; 3, severe; 4, life threatening.

TABLE IV

Hematologic Toxicity

No. of courses	No. in which toxicity occurred	5-Azacytidine dose	Nadir count (10^3)	Leukocytes (day of nadir)	Day count normal	Comments
7	2	50 mg/m²/d×5	3.0	19	30	
			3.3	21	31	
6	none	100 mg/m²/d×5				
5	2	133 mg/m²/d×5	3.9	18	26	this patient also developed thrombocytopenia day 9 (38,000): platelets returned to normal on day 24
			2.95	19	22	
3	3	158 mg/m²/d×5	2.0	12	22	
			2.1	14	24	
			1.3	11	21	
No. of pts.						
4	1	200 mg/m²/wk	3.8	11	22	Count returned to normal while receiving 5-azacytidine
4	1	400 mg/m²/wk	0.7	12	36	
5	2	533 mg/m²/wk	3.9	33	46	
			3.7	35	48	
2	2	633 mg/m²/wk	4.7	28	32	
			3.4	34	40	

72 units. This patient was receiving compazine for control of his emesis.

Hematologic toxicity became more evident with increasing dose levels at both the daily and weekly schedules. On the weekly schedule, a cumulative effect was suggested by a later nadir of leukocyte depression and a longer period before a return to normal occurred. Only one instance of thrombocytopenia occurred at the the doses studied (Table IV).

Discussion

5-Azacytidine has been studied by a number of investigators to delineate its clinical pharmacology and therapeutic activity. It is apparent from the animal studies that the total dose required to produce toxicity is less when the drug is given intermittently over a long period of time rather than in a short course of therapy. In our studies in humans, the toxic side effects (gastrointestinal), which were the dose-limiting factors, were seen in patients receiving both the five-day course and the once-weekly schedule without much difference in severity. Patients receiving the once-weekly schedule, however, were more likely to be willing to continue therapy because the gastrointestinal side effects were of shorter duration and there was a period during which they were free of these toxic side effects before the next scheduled dose.

Since the primary problem with drug administration, as far as the patient is concerned, is vomiting, nausea, and at times, diarrhea, it may be worthwhile to give the drug on a weekly schedule dividing the dose into two injections, 12 hours apart. Some of our preliminary studies suggest that the gastrointestinal side effects may be diminished when drug is given in this manner or when the total dose is given in two divided doses three to four days apart.

The tolerated daily dose for this study was in the range of 150 mg/m^2 given for five days every two weeks. The dose-limiting factors were severe nausea and vomiting with associated weight loss. For the single weekly dose, 500 mg/m^2 appeared to be the tolerated dose without the severe nausea, vomiting, and diarrhea of the daily schedule. This dose divided into two injections given 12 hours apart could be utilized in the phase I trials or for combination chemotherapy studies. No attempt was made to study the therapeutic efficacy of the drug in this short-range phase I evaluation.

In other clinical studies, this drug has been administered to patients with both solid tumors and hematologic malignancies. The successful treatment of patients with acute leukemias refractory to other forms of therapy has been reported by two investigators.[4,10] Bellet et al.[9] reported objective tumor regressions in two patients given subcutaneous 5-azacytidine, one adenocarcinoma of the breast and one adenocarcinoma of the ovary. Much less success has been seen in patients with solid tumors.[2,8]

Clinical studies to date have indicated that 5-azacytidine has some activity against myelocytic and lymphocytic leukemia in children, has only very limited activity in adenocarcinoma of the breast and adenocarcinoma of the ovary and malignant melanoma, and no activity in advanced gastrointestinal carcinoma. Whether this drug has any value in combination chemotherapy is yet to be established.

Summary

5-Azacytidine was administered daily to 12 patients in a five-day schedule and to 15 patients in a weekly schedule as part of a phase I trial. The daily dose ranged from 50 mg/m^2 to 158 mg/m^2 and the weekly dose, from 200 mg/m^2 to 633 mg/m^2. The maximum total dose was 2000 mg in the daily schedule and 3775 mg in the weekly schedule. The major toxicity

was gastrointestinal, with nausea and vomiting occurring in all patients in this study. Myelosuppression was less frequently encountered and appeared to be related to the increase in 5-azacytidine dose. Patients receiving 5-azacytidine in a weekly schedule of administration appeared to tolerate the drug better and to be more willing to continue their therapy.

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Randomized Controlled Trial of Azacitidine in Patients With the Myelodysplastic Syndrome: A Study of the Cancer and Leukemia Group B

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Purpose: **Patients with high-risk myelodysplastic syndrome (MDS) have high mortality from bone marrow failure or transformation to acute leukemia. Supportive care is standard therapy. We previously reported that azacitidine (Aza C) was active in patients with high-risk MDS.**

Patients and Methods: **A randomized controlled trial was undertaken in 191 patients with MDS to compare Aza C (75 mg/m^2/d subcutaneously for 7 days every 28 days) with supportive care. MDS was defined by French-American-British criteria. New rigorous response criteria were applied. Both arms received transfusions and antibiotics as required. Patients in the supportive care arm whose disease worsened were permitted to cross over to Aza C.**

Results: **Responses occurred in 60% of patients on the Aza C arm (7% complete response, 16% partial response, 37% improved) compared with 5% (improved) receiving supportive care ($P < .001$). Median time to leukemic transformation or death was 21 months for Aza C versus 13 months for supportive care ($P = .007$). Transformation to acute myelogenous leukemia occurred as the first event in 15% of patients on the Aza C arm and in 38% receiving supportive care ($P = .001$). Eliminating the confounding effect of early cross-over to Aza C, a landmark analysis after 6 months showed median survival of an additional 18 months for Aza C and 11 months for supportive care ($P = .03$). Quality-of-life assessment found significant major advantages in physical function, symptoms, and psychological state for patients initially randomized to Aza C.**

Conclusion: **Aza C treatment results in significantly higher response rates, improved quality of life, reduced risk of leukemic transformation, and improved survival compared with supportive care. Aza C provides a new treatment option that is superior to supportive care for patients with the MDS subtypes and specific entry criteria treated in this study.**

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MYELODYSPLASTIC syndrome (MDS) represents a heterogeneous hematopoietic disorder in which mature blood cells are derived from an abnormal multipotent progenitor cell. The disease is characterized by morphologic features of dyspoiesis, a hyperproliferative bone marrow, and peripheral-blood cytopenias involving one or more lineages.[1] Refractory anemia (RA) with or without ringed sideroblasts can persist for years, but RA with excess blasts (RAEBs) or RAEBs in transformation to leukemia (RAEB-T) exhibit an accelerated course.[2-5] Most patients with high-risk MDS (ie, French-American-British [FAB] subtypes with excess blasts) die within 1 year from progressive bone marrow failure attributable to hemorrhage or infection. In 35% to 40% of patients, transformation to acute leukemia occurs, which is often refractory to present therapy.[1]

Therapies tried for MDS include granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), erythropoietin, and chemotherapy.[6-22] None has altered the natural history of the disease. Supportive care with antibiotics and transfusions is considered the standard of care. Allogeneic bone marrow transplantation, a potentially curative approach, is a realistic option for only approximately 5% of patients.[23-28]

Azacitidine (Aza C), a pyrimidine nucleoside analog, was developed as an antitumor agent.[29-31] In addition to cytotoxic effects, it induces differentiation of malignant cells in vitro.[32-35] Aza C inhibits DNA methyltransferase, the enzyme in mammalian cells responsible for methylating newly

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Table 1. Additional Eligibility Criteria for Bone Marrow Dysfunction in Patients With RA and RARS*

RBC	Symptomatic anemia requiring RBC transfusions for at least 3 months before study entry
Platelets	Thrombocytopenia with two or more platelet counts $\leq 50 \times 10^9$/L or a significant clinical hemorrhage requiring platelet transfusions
WBC	Neutropenia with an ANC $< 1 \times 10^9$/L and an infection requiring intravenous antibiotics

*Patients had to meet at least one of these criteria.

Table 2. Eligibility Criteria

- Age > 15 years
- Life expectancy ≥ 2 months
- Performance status ≤ 2 (NCI scale, 0-4)
- Total bilirubin ≤ 1.5 × ULN
- AST/ALT ≤ 2 × ULN
- Serum creatinine ≤ 1.5 × ULN
- Serum CO_2 ≥ 19 mEq/L
- No previous treatment for MDS with Aza C, G-CSF, GM-CSF, or other hematopoietic cytokines (except for erythropoietin)
- No erythropoietin, corticosteroids, interferon, or retinoids within 1 month before study
- No prior history of leukemia
- No pregnancy on uncontrolled congestive heart failure

Abbreviations: NCI, National Cancer Institute; ULN, upper limit of normal.

synthesized DNA, resulting in synthesis of hypomethylated DNA and changes in gene transcription and expression.[32-34] In patients with beta-thalassemia or sickle-cell anemia, Aza C caused hypomethylation of the gamma globin chain gene with increased production of fetal hemoglobin.[36-38]

The Cancer and Leukemia Group B (CALGB) conducted a phase II study of Aza C administered to 43 hospitalized patients as a continuous intravenous infusion for 7 days every 28 days for 4 months.[39] Responses (complete response [CR], partial response [PR], or improved) occurred in 49% of patients with high-risk MDS (RAEB and RAEB-T). A second phase II study of 67 patients with high-risk MDS showed that Aza C as a subcutaneous daily bolus injection at the same dose and schedule on an ambulatory basis produced comparable results in response rate, response duration, and survival.[40] The present phase III randomized trial compares subcutaneous Aza C treatment with supportive care.

PATIENTS AND METHODS

Patient Selection

All patients fulfilled the FAB classification criteria for MDS.[41-43] Patients with RA or RA with ringed sideroblasts (RARS) met additional criteria of significant marrow dysfunction (Table 1). Bone marrow aspiration and biopsy were required within the 2 weeks before registration. Peripheral-blood films and marrow specimens were independently evaluated through centralized pathology review (D.N.).

Patients with therapy-related MDS were eligible if they were cancer-free for at least 3 years and had not received radiation or chemotherapy for 6 months. Additional eligibility requirements are listed in Table 2. The protocol was approved by the institutional review boards of all participating institutions. Each patient provided written informed consent.

Treatment Regimen

Patients were stratified by FAB subtype and randomly assigned to supportive care or Aza C. The use of all hematopoietic growth factors was prohibited. Transfusions and antibiotics were administered as required. Marrow samples were obtained before study entry, at day 57, and at day 113.

Aza C arm. Aza C (75 mg/m^2/d) was injected subcutaneously in 7-day cycles beginning on days 1, 29, 57, and 85. Aza C, supplied by the National Cancer Institute (Bethesda, MD) in vials of 100 mg of powder plus 100 mg of mannitol, was suspended in 4 mL of sterile water and injected as a slurry with a maximum volume of 4 mL per injection site. If a beneficial effect was not demonstrated by day 57 and no significant toxicity other than nausea or vomiting had occurred, the dose of Aza C was increased by 33%. Once benefit occurred on a particular dosage, Aza C was continued unless toxicity developed. Patients were assessed after the fourth cycle. Those who achieved CR continued on Aza C for three more cycles; those with PR or improvement continued on Aza C until either CR or relapse occurred. Responses were initially evaluated by the treating physician but subsequently were scored independently by two experienced investigators (L.R.S. and R.M.S.) to validate responses. Patients who progressed (see Definitions, below) during the induction phase and those with stable disease at day 113 were classified treatment failures and removed from treatment.

Supportive care arm. After a minimum interval of 4 months of supportive care, patients whose disease was worsening (see Definitions, below) were permitted to cross over to Aza C treatment. Patients could exit supportive care before 4 months but only because of death, withdrawal of consent, transformation to acute leukemia, or a platelet count persistently less than 20×10^9/L after week 8. Patients transforming to acute myelogenous leukemia (AML) exited at any time; those with less than or equal to 40% blasts in the marrow crossed over to Aza C, whereas those with greater than 40% blasts received other treatments.

Cross-over. All data necessary to establish eligibility for cross-over from supportive care to Aza C were independently reviewed by the study chair, whose prior approval was required before cross-over (n = 46 of 49). Cross-over patients were studied and treated identically to patients initially randomized to Aza C.

Quality-of-life assessment. Quality of life (QOL), an integral component of the study, was systematically assessed during standard telephone interviews by one of two trained nurses (E.P.D. or R.O.R.) before randomization and on days 50, 106, and 182. The QOL battery included measures of four dimensions: physical symptoms and functioning, psychological state, social functioning, and sociodemographic characteristics. The questionnaire consisted of two validated scales, the European Organization for Research and Treatment of Cancer (EORTC) QOL and the Mental Health Inventory (MHI). Questionnaires were given or mailed to patients before the telephone interviews; the interview methodologies have been validated in prior CALGB studies.[44]

Table 3. Response Criteria

Trilineage response	≥ 50% restitution of the initial deficit from normal in all three peripheral-blood cell counts and elimination of all blood transfusion requirements		
Monolineage or bilineage response	≥ 50% restitution of the initial deficit from normal in one or two peripheral blood cell counts		
	CR	PR*	Improved†
Bone marrow	M_0 or M_1	≤ 50% of initial bone marrow blasts	—
Peripheral blood			
Counts	H_0	Trilineage response	Monolineage or bilineage response
Blasts	0	0	—
Transfusion	0	0	≤ 50% of baseline
Relapse	> 5% bone marrow blasts	>30% bone marrow blasts	Return to pretreatment blood values or return of RBC or platelet transfusion requirement§
	or		
	Return to pretreatment blood values or return of RBC or platelet transfusion requirement‡		

Abbreviations: M_0, normal bone marrow; M_1, < 5% blasts in the bone marrow, some dyshematopoietic features may persist; H_0, complete normalization of the peripheral-blood counts (ie, hemoglobin ≥ 133 g/L [males], hemoglobin ≥ 117 g/L [females]; WBC ≥ 4.4 × 10^9/L; ANC 1.8 × 10^9/L platelets ≥ 140 × 10^9/L).

*Peripheral-blood criteria alone were used for potients with RA and RARS.

†Criteria for improvement are satisfied by either monolineage or bilineage response or ≥ 50% decrease in transfusion requirement from baseline.

‡For patients with RA or RARS, relapse could be defined on peripheral-blood criteria alone.

§Changes in blood counts secondary to drug-induced myelosuppression did not constitute criteria for relapse.

Definitions

Response criteria are outlined in Table 3.

Relapse of responders. Relapse from CR was defined as greater than 5% myeloblasts in the bone marrow. Relapse from a PR was defined as ≥ 30% myeloblasts in the bone marrow (except for patients with RA and RARS, where peripheral-blood criteria alone or in conjunction with the bone marrow were used). Relapse for improved patients was defined as a decline to pretreatment levels in the blood counts, which were the criteria for response, or the recurrence of a transfusion requirement secondary to disease progression. Reversible changes in blood counts secondary to drug-induced myelosuppression did not constitute criteria for relapse.

Treatment failure in nonresponders. Treatment was considered to have failed in nonresponders receiving supportive care if they advanced to a higher FAB subtype (ie, to RAEB or RAEB-T) or to AML, remained RBC transfusion–dependent before and during study, or developed progressive bone marrow failure, defined as the following: (1) confirmed fall from baseline of greater than 25% in all three peripheral-blood cell lines or greater than 50% fall in two cell lines or a greater than 75% fall in one cell lineage or (2) development of a RBC transfusion requirement (ie, in patients not receiving RBC transfusions before study entry, if the hemoglobin fell to < 9 g/L in patients > 65 years of age or to ≤ 8 g/L if ≤ 65 years of age). Supportive care treatment was also considered to have failed if patients had a platelet count persistently lower than 20 × 10^9/L after week 8 (N = 9). Nonresponders taking Aza C were evaluated identically for treatment failure, and when treatment failure was present, these patients exited protocol study but were followed for survival.

Statistical Methods

Four analyses, three interim and one final, were planned using O'Brien-Fleming stopping rules. The first three analyses found a significant difference in response between the arms (undisclosed information), but the Data and Safety Monitoring Board recommended continuation of the study so that QOL, survival, and transfusion requirements could be studied in a larger sample. Twenty-six percent of the patient records were independently audited by the CALGB Data Audit Committee for protocol compliance and data quality.

Analyses were performed on an intention-to-treat basis. Patients (n = 20) determined by central pathology review to have acute leukemia at entry were noninformative for AML transformation and the time-to-treatment failure analyses. Response rates of the randomized arms were compared with the χ^2 test of proportions. Survival, time to response, and response duration were estimated with the Kaplan-Meier method and compared with the log-rank test.[45,46] In testing for differences in survival and time to transformation to AML, randomized induction treatments were compared and cross-over was ignored. The two-stage statistical methodology recommended by Gelman et al[47] was used in analyzing the time to AML to account for the competing risk of death.

Prestudy RBC transfusion requirements (present/absent) were calculated. RBC transfusion data were standardized to the number of units per month and the means across time. Differences in transfusion requirements could have been influenced by the loss of patients because of death, cross-over, and dropout (attrition bias) and by physician discretion in the administration of transfusions.

Times to initial response and to best response were measured from study entry to the date that initial and best response criteria were met, respectively. Duration of response was measured from initial response to relapse. Time to treatment failure was measured from study entry to the point of relapse (for responders) or failure (for nonresponders). The time from study entry to transformation to AML or death was chosen as the most meaningful clinical end point, because survival and QOL decline rapidly for patients with MDS after AML develops.

QOL Analysis

A pattern-mixture model was used to examine treatment differences in QOL over time.[48-51]

Table 4. Demographic and Clinical Characteristics at Study Entry

	Aza C		Supportive Care		Total	
	No. of Patients	%	No. of Patients	%	No. of Patients	%
Randomized	99	52	92	48	191	
FAB classification						
RA	17	17	20	22	37	19
RARS	5	5	3	3	8	4
RAEB	32	32	34	37	66	35
RAEB-T	27	27	18	20	45	24
CMMoL	7	7	7	8	14	7
Other*	11	11	10	11	21	11
IPSS risk group†						
Low	2	2	5	6	7	9
Intermediate-1	21	26	16	20	37	45
Intermediate-2	9	11	13	16	22	27
High	7	9	8	10	15	19
Age						
Median	69		67		68	
Range	31-92		35-88		31-92	
Sex						
Male	72	73	60	65	132	69
Female	27	27	32	35	59	31
Prior radiation therapy	8	8	3	1	11	6
Prior chemotherapy	15	15	12	13	27	14
Prior treatment for MDS	16	16	17	18	33	17
Infection requiring treatment	6	6	4	4	10	5
Active bleeding	16	16	18	20	34	18
Patients requiring platelet transfusions‡	18	18	10	11	28	15
Patients requiring RBC transfusions‡	68	69	56	61	124	65
Time from diagnosis to study entry						
Median	77 days		87 days			
Range	1 day-6.4 years		2 days-6 years			

Abbreviation: IPSS, International Prognostic Scoring System.

*Includes 19 AML, one unclassifiable acute leukemia, and ane undefined MDS.

†Complete cytogenetic data to determine the IPSS score were only available for 81 patients.

‡During the 3 months preceding study entry.

RESULTS

Patient Characteristics

One hundred ninety-one patients with MDS deemed eligible by treating investigators were entered on CALGB 9221 between February 1994 and May 1996 from 26 main member institutions and their 30 affiliated hospitals. Response and toxicity were analyzed on data available through February 1999. After central pathology review, 20 patients were determined to have AML at study entry. These patients are excluded only from the AML transformation and time to progression analyses. The conclusions were unchanged if these patients were excluded from all analyses (data not shown).

The two arms were evenly balanced at study entry (Table 4). There were no differences by FAB subtype, cytogenetic analysis (n = 81), International Prognostic Scoring System score,[52] or time from diagnosis to study entry. Hematologic parameters at study registration are described in Table 5.

Analysis of Response

Among patients randomized to supportive care, 5% (n = 5) met the criteria for improvement. No patient achieved a CR or PR (Table 6). All five patients categorized as improved either had a rising WBC count or absolute neutrophil count (n = 4) or platelets (n = 1) in the process of transforming from MDS to AML. On the Aza C arm, 60% (n = 60) responded ($P < .0001$), with 7% (n = 7) achieving CR, 16% (n = 16) having PR, and 37% (n = 37) improving. In no case was improvement of cytopenia a component of transformation to AML. The trilineage response was 23% for Aza C and 0% for supportive care. Among the 37 Aza C patients categorized as improved, 35% had increases in all three cell lines (but insufficient to meet criteria for PR), 30% had increases in two cell lines, and 35% had an increase in only a single cell line (Fig 1). Response to Aza C was independent of MDS classification. Responses for patients with RA and RARS (9% CR [n = 2];

Table 5. Hematologic Parameters at Study Entry

	Aza C	Supportive Care	Total
Hemoglobin,* g/L			
Median	90	93	91
Range	53-140	57-140	53-140
WBC, × 10⁹/L			
Median	3.6	3.7	3.7
Range	0.7-124.5	0.4-41.2	0.4-124.5
ANC, × 10⁹/L			
Median	1.5	1.7	1.6
Range	0.04-90.9	0.1-27.6	0.04-90.0
Platelets, × 10⁹/L			
Median	52	72	63
Range	4-479	4-570	4-570

Abbreviation: ANC, absolute neutrophil count.
*Median hemoglobin levels at study entry may reflect transfused values.

18% PR [n = 4]; 32% improved [n = 7]) were comparable with patients with RAEB, RAEB-T, and chronic myelomonocytic leukemia (CMMoL) (8% CR [n = 5], 15% PR [n = 10], 38% improved [n = 25]) among patients classified according to central pathology review (Table 4). Median times to initial response and best response were 64 and 93 days, respectively. The median duration of response among patients who achieved CR, PR, or improvement was 15 months (95% confidence interval [CI], 11 to 20 months) (Fig 2).

Of 49 patients who crossed over from supportive care to Aza C, 47% (n = 23) then responded, with 10% (five patients) achieving CR, 4% (two patients) having PR, and 33% (16 patients) improving. The trilineage response was 14%. Neither age nor sex influenced response rates.

Time to Treatment Failure

With certain exceptions (see above), the study design intended that patients remain on the initial randomization arm for a minimum of 4 months. The median time to exit from supportive care (ie, median time to treatment failure) was 3.8 months (95% CI, 3.5 to 4.0 months; range, 0.6 to > 55 months); the median time to exit from the Aza C arm was 9.1 months (95% CI, 5.6 to 11 months; range, 0.1 to > 44 months) ($P < .0001$).

Analysis of Time-to-AML Transformation or Death

The effects of treatment on transformation to AML or death are illustrated in Fig 3. The median time to event for supportive care was 12 months (95% CI, 8 to 15 months) compared with 21 months (95% CI, 16 to 27 months) for Aza C ($P = .007$). For patients with high-risk FAB subtypes (RAEB, RAEBT, or CMMoL), the median time to AML or death for supportive care was 8 months (95% CI, 4 to 13 months) compared with 19 months (95% CI, 13 to 21 months) for Aza C ($P = .004$). There were an insufficient number of events to estimate medians in the patients with low-risk FAB. Overall, FAB subtype was a significant predictor of time to AML or death ($P = .0003$).

Transformation to AML occurred as the first event in 15% of the patients randomized to Aza C compared with 38% of patients randomized to supportive care ($P = .001$). Indeed, during the first 6 months after study entry, 3% of patients taking Aza C transformed to AML while 24% of patients on supportive care transformed ($P < .0001$). Of the patients who transformed to AML in the supportive care group, 77% were diagnosed at study entry as having RA/RARS or RAEB but not RAEB-T. Figure 4 represents the percent bone marrow myeloblasts at study entry compared with the percentage of blasts in the marrow or peripheral blood (National Cancer Institute criteria) at the time of transformation. To demonstrate the biologic impact on survival of the transformation to leukemia, we performed a landmark analysis after a 12-month date of the association of transformation to AML with survival. The two subgroups included 13 patients who had already transformed to AML by the landmark date and 93 patients who had not yet transformed, both groups independent of therapy. Patients who died before 12 months were excluded. The median additional survival (after the 12-month landmark) was 3 months (95% CI, 1 to 11 months) for patients who had already transformed and 18 months (95% CI, 14 to 26 months) for patients who had not yet transformed to AML ($P < .001$).

Table 6. Analysis of Response

	Aza C		Supportive Care		Cross-Over	
	No. of Patients	%	No. of Patients	%	No. of Patients	%
No. evaluated	99		92		49	
CR	7	7*	0	0	5	10
PR	16	16*	0	0	2	4
Improved	37	37*	5	5	16	33
Total	60	60*	5	5	23	47

*Significant differences between the arms in CR rate ($P = .01$), CR + PR rate ($P < .0001$), and CR + PR + improvement rate ($P < .0001$) were observed.

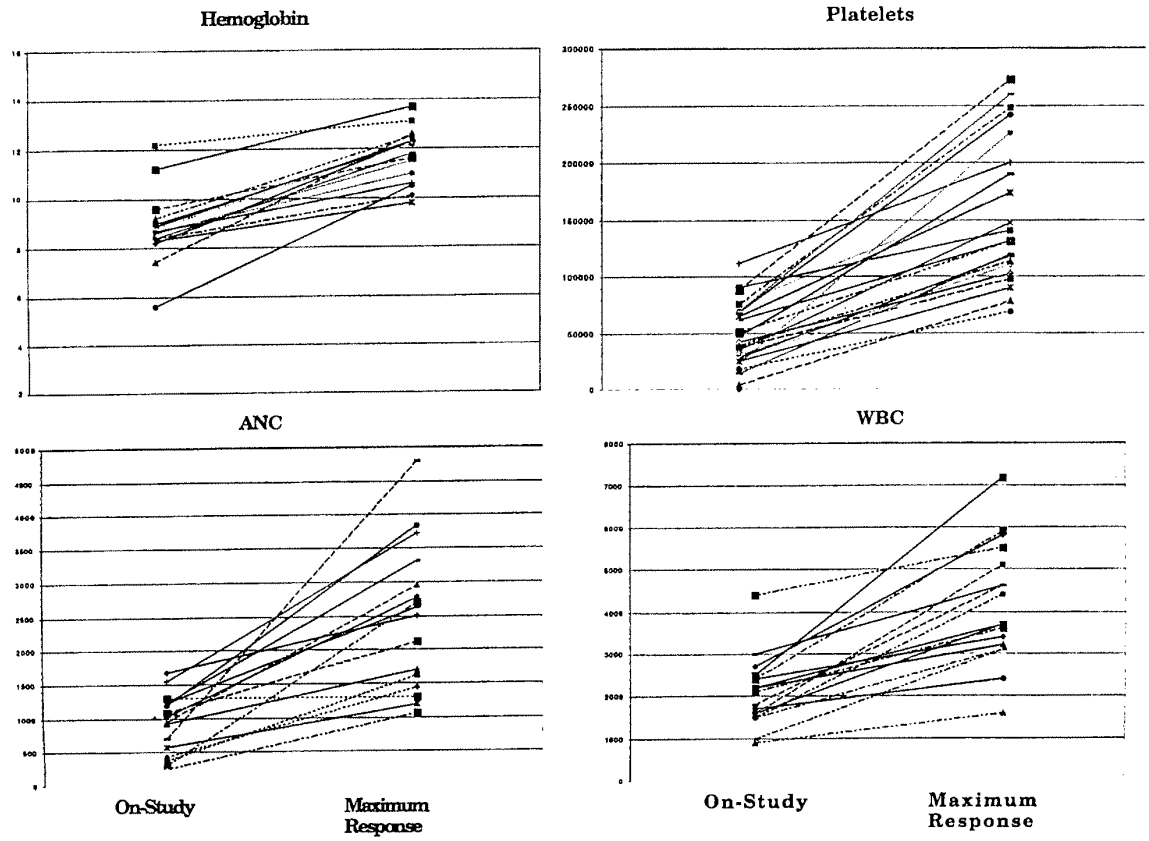


Fig 1. Changes in peripheral-blood counts at the time of response compared with study entry for 37 patients in the Aza C arm classified as improved. Patients who achieved CR or PR are not included.

Effects on RBC and Platelets

The mean number of RBC transfusions increased for the patients taking Aza C in the first month of treatment but thereafter declined, whereas the mean number of transfusions remained stable or increased for patients on supportive care. By definition (Table 3), patients achieving CR or PR had an elimination of RBC or platelet transfusion requirements. Among the 37 patients improved, 73% had an RBC response, 35% (n = 13) had a 50% or greater restitution in the RBC deficit (lineage response), 22% (n = 8) had an elimination of all RBC transfusion requirements, and 16% (n = 6) had a decrease by 50% or greater in RBC transfusions. Thus, among the 99 patients randomized to Aza C, 51% had an RBC lineage response. Among the 65 patients receiving RBC transfusions at study entry, 29 (45%) had an elimination of all transfusions and another six (9%) had a reduction in transfusions by 50%. In addition, lineage responses for platelets and WBC occurred in 47% and 40%, respectively, among those treated with Aza C.

Effects of Treatment on QOL

Patients on the Aza C arm experienced significantly greater improvement over time in fatigue (EORTC, P = .001), physical functioning (EORTC, P = .002), dyspnea (EORTC, P = .0014), psychosocial distress (MHI, P = .015), and positive affect (MHI, P = .0077) than patients in the supportive care group. Significant differences persisted after controlling for RBC transfusions. Before cross-over, the QOL of patients on supportive care was stable or worsening. After cross-over to Aza C, significant improvements occurred in fatigue (EORTC, P = .0001), physical functioning (EORTC, P = .004), dyspnea (EORTC, P = .0002), and general well-being (MHI, P = .016).[50] A complete report of QOL will be presented elsewhere.[51]

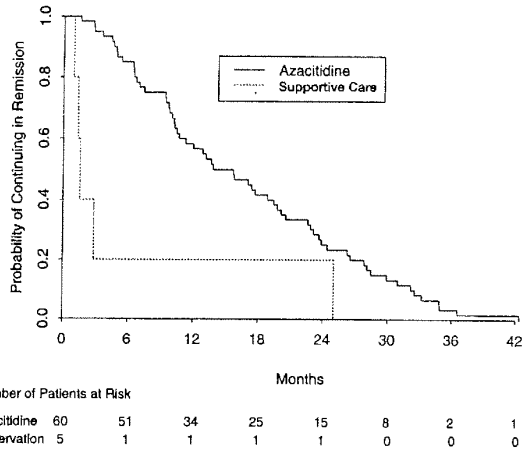


Fig 2. Duration of response. Measured from time of initial response to relapse in patients with CR, PR, or improvement and estimated according to the method of Kaplan-Meier.

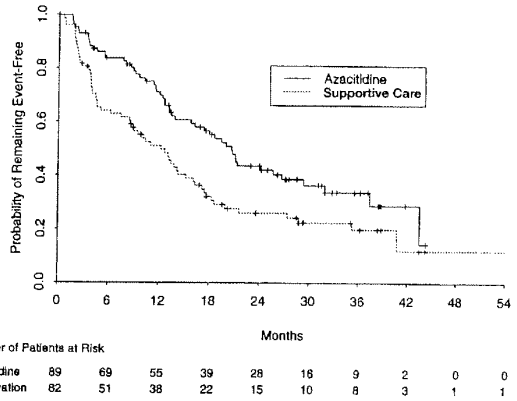


Fig 3. Time to AML transformation or death. Measured from entry on study to the time of first event, either transformation to AML or death, and estimated according to the Kaplan-Meier method.

Detailed analyses make unlikely placebo or Hawthorne[53] effects as explanations for improvements in QOL by Aza C.

Overall Survival

The median survival was 20 months (95% CI, 16 to 26 months) for patients randomized to Aza C compared with 14 months (95% CI, 12 to 14 months) for patients undergoing supportive care (53% of whom received Aza C after cross-over) ($P = .10$)(Fig 5). To eliminate the confounding effect caused by including the 49 cross-over patients in the survival analysis, a landmark analysis was done in which the survival of three subgroups of patients were compared from a 6-month landmark date. These subgroups were supportive care patients who never crossed over or who crossed over only after 6 months, supportive care patients who crossed before 6 months, and patients who were initially randomized to Aza C. The 36 patients who died before the landmark date were excluded. The median survival (after the 6-month landmark date) for these three groups was 11, 14, and 18 months, respectively (Fig 6). The Aza C group was significantly different from the supportive care subgroup who crossed over late or never ($P = 0.03$). Supportive care patients who crossed over early (subgroup 2) had a longer median survival than the patients who crossed over late or never (subgroup 1), although this did not reach statistical significance ($P = .11$). Survival by treatment arm and FAB risk group is demonstrated in Fig 7.

Treatment-Related Toxicity

The most common toxicity of Aza C was myelosuppression. In patients with severe cytopenias from their disease,toxicity was difficult to assess, rendering useless the standard criteria used for hematologic toxicity where the pretreatment marrow is normal. On the basis of standard CALGB criteria, grade 3 or 4 leukopenia occurred in 59%, granulocytopenia in 81%, and thrombocytopenia in 70% of patients receiving Aza C. When hematologic toxicity was reassessed centrally using relative changes in peripheral-blood counts compared with those at study entry, a decrease of 50% to 74% was defined as grade 3 and 75% or greater was defined as grade 4. Based on these criteria, grade 3 or 4 leukopenia occurred in 43%, granulocytopenia in 58%, and thrombocytopenia in 52% of patients receiving Aza C. Toxicity was transient, and patients usually recovered in time for the next treatment cycle. Infection was thought to have been related to treatment in 20% of patients. Nausea or vomiting occurred in 4%. There was one ($\leq$ 1%) treatment-related death.

DISCUSSION

The present results confirm our earlier observations of the beneficial effects of Aza C on bone marrow function in high-risk MDS and extend these findings to symptomatic RA and RARS. The same stringent response criteria used in our original trials of Aza C, developed and defined in the absence of standardized criteria, were used in the present study.[39] The 5% response rate in the supportive care arm indicates that the criteria are sufficiently robust to filter out ordinary variation in blood counts. Incremental changes in peripheral-blood counts among patients improved were sizable (Fig 1). Thus, patients were not categorized as

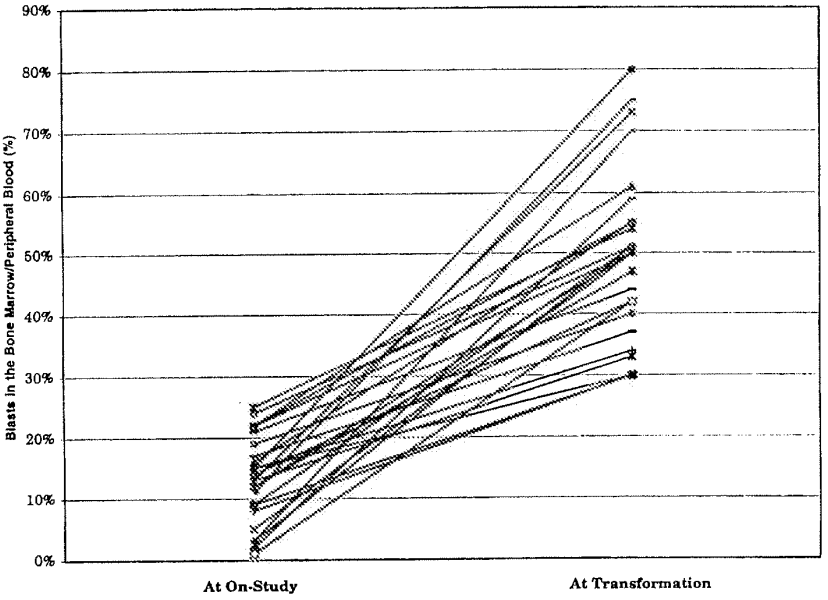


Fig 4. Comparison of myeloblasts in the bone marrow or peripheral blood at study entry and the time of leukemic transformation.

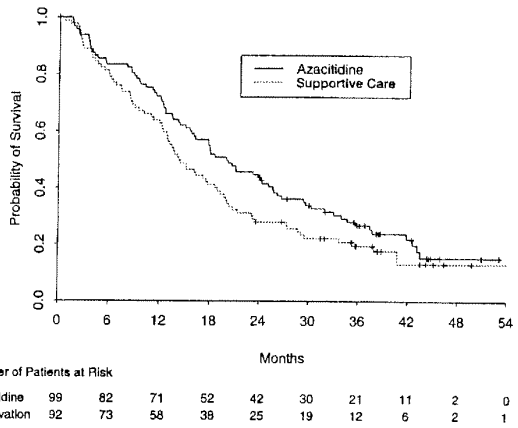
improved on the basis of only a marginal increase in counts as a potential byproduct produced by a quirk of the response criteria. The 60% response rate for Aza C shows that the criteria are sensitive and specific enough to detect biologically important changes, because they are associated with prolonged survival and improved QOL. Our patients were treated at 26 academic centers and 30 of their community affiliates. Thus, our results may predict general medical community achievement.

The number of deaths in the two arms in the first 4 months of study was comparable. The frequency of transformation to leukemia for patients on supportive care was eight-fold higher than patients treated with Aza C in the first 6 months from study entry. Over the entire course of the study, the rate was 2.5-fold higher, the lesser frequency possibly reflecting the fact that many patients were receiving Aza C after cross-over. Differences between the arms in leukemic transformation could not be explained by FAB subtype, International Prognostic Scoring System scores, or time from diagnosis to study entry. Time to leukemic transformation or death represents the purest and most objective manifestation of disease progression for MDS. The landmark analysis demonstrates that transformation to AML has a significantly adverse effect on survival. Aza C delays and decreases transformation to acute leukemia. This is the first description of a drug with this capacity.

The effect of initial treatment with Aza C on overall survival was confounded by the fact that 49 supportive care patients were crossed over to Aza C during their survival follow-up. The landmark analysis diminishes the confounding effect and demonstrates a significant survival advantage in favor of those treated with Aza C initially compared with those not treated or who received treatment only after 6 months of supportive care (Fig 6). A salvage benefit may nonetheless still be important even for patients treated late in the course of their disease.

Significant improvements in QOL, particularly for fatigue, physical functioning, dyspnea, and general well-being, were derived from Aza C treatment, even in the supportive care patients after cross-over. The data indicate that Aza C treatment is more effective in improving QOL than simply raising hemoglobin values with RBC transfusions.

Aza C appears to be superior to other drugs that have been used for MDS. Agents that can induce hematopoietic differentiation in vitro have been extensively tested, and 13-*cis*- and all-*trans*-retinoic acid, 1,25-dihydroxy vitamin D_3, butyrate, cytarabine, and hexamethylene bisacetamide have produced feeble clinical responses. Amifostine has produced responses, but its activity has yet to be fully defined.[54] None of these drugs have caused significant trilineage responses, sustained remissions, or prolonged



Number of Patients at Risk

Azacitidine	99	82	71	52	42	30	21	11	2	0
Observation	92	73	58	38	25	19	12	6	2	1

Fig 5. Overall survival by randomized arm and estimated according to the Kaplan-Meier method. Patients who were initially in the supportive care group and crossed over to treatment with azacitidine are included in the supportive care group in this plot.

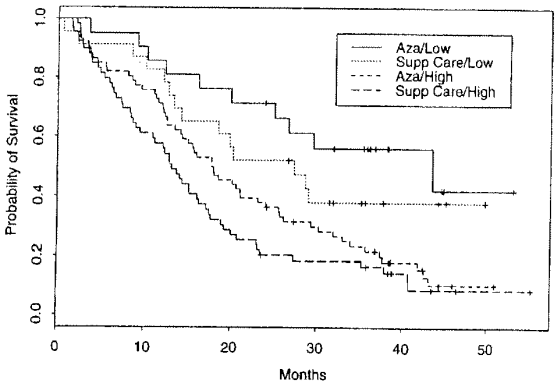


Fig 7. Survival by randomized arm and FAB subtype. FAB subgroups were divided into low-risk (RA/RARS) and high-risk (RAEB, RAEB-T, or CMMoL) groups. Median survival: Aza/Low, 44 months; supportive care (SC)/Low, 27 months; Aza/High, 18 months; SC/High, 13 months.

survival.[55-65] Aggressive antileukemic type therapy and newer agents such as topotecan alone or in combination have produced response rates up to 65% but have not been reported to alter the disease outcome.[66-71]

Four randomized controlled trials have been previously conducted in patients with MDS. *Cis*-retinoic acid demonstrated no advantage compared with placebo.[55,72] Low-dose cytarabine (10 mg/m^2 every12 hours) compared with supportive care led to a 35% hematologic response (using less stringent criteria than the present study) but no differences in time to progression, frequency of transformation to AML, or survival.[22,73] Filgrastim (G-CSF) was compared with supportive care. The death rate for patients with RAEB and RAEB-T was significantly accelerated by G-CSF, with a median survival of 10 months compared with 21 months for supportive care, leading to early termination of the study.[74] Treatment with sargramostim (GM-CSF) resulted in increases in myelomonocytic and lymphoid lineages, with a decrease in frequency of infections in those treated. There were no effects on platelets or red cells. Impact on outcome has not been reported.[11]

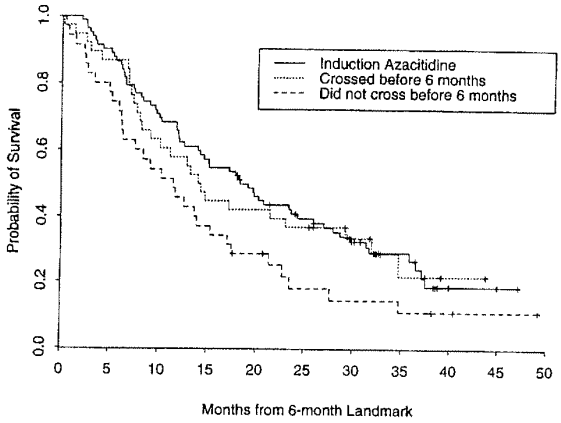


Fig 6. Survival from landmark date by cross-over status (Kaplan-Meier method). Patients were subgrouped as supportive care patients who either never crossed over or crossed over after 6 months, supportive care patients who crossed over before 6 months, ond patients who were initially randomized to Aza C.

The mechanism by which Aza C produces its effects is most likely multifactorial. Aza C can produce significant myelosuppression, particularly at higher doses. The doses used in this study and the two prior phase II studies produced marrow hypoplasia in only 10% of patients. Nevertheless, myelosuppression leading to lower peripheral-blood counts and increased RBC transfusion requirements occurred during the first cycle of treatment. Continued treatment without dose reduction led to improved bone marrow function in most patients. Prolonged treatment may have inhibited the MDS clone, permitting residual normal hematopoiesis to emerge. Conversely, Aza C might have exerted a cytotoxic effect on regulatory T cells or other modulatory cells that were inhibiting hematopoiesis.

Aza C could also be acting as a biologic response modifier. The response of hematopoietic progenitors to cytokines is impaired in patients with MDS.[75] This may be attributable in part to abnormalities of the signal transduction pathway downstream from the cytokine receptors.[76-79] In vitro data suggest that Aza C can modulate the cytokine signal transduction pathway, rendering sensitive unrespon-

sive cells to the effects of cytokines, partially restoring normal hematopoietic regulation.[80-82]

As observed in our prior studies, most responding patients demonstrated response beginning in the third or fourth month. This is consistent both with a low-dose cytotoxic effect and with Aza C acting as a biologic response modifier. Incorporation of Aza C into DNA inhibits DNA methyltransferase and induces DNA hypomethylation.[32,83-86] This effect is S-phase dependent, and two or more cycles of DNA synthesis are required to alter gene transcription and expression.[32,84-86] Thus, repetitive exposure on the present low-dose intermittent schedule may have affected small numbers of cells during each treatment, requiring three to four cycles before the effects became clinically apparent. Alteration in the methylation of the *p15* gene has been implicated in transformation of MDS to AML and could be modulated by Aza C, thus reducing risk of leukemic transformation.[87,88]

Although Aza C is active in the present regimen, other doses and schedules might improve its efficacy. Additional studies of Aza C should build on these results. Besides optimization of dose and schedule, combinations with cytokines and other agents that modulate signal transduction are logical areas of exploration. The present study demonstrates that Aza C is effective therapy for patients with MDS with the subgroups and profiles treated in this study. Aza C improves their bone marrow function, decreases and delays significantly transformation to AML, and improves QOL and survival compared with supportive care. These data suggest that Aza C should be considered the treatment of choice for patients with MDS who meet the entry criteria stipulated in this study.

APPENDIX

The appendix listing participating institutions and investigators is available online at www.jco.org.

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ORIGINAL REPORT

Further Analysis of Trials With Azacitidine in Patients With Myelodysplastic Syndrome: Studies 8421, 8921, and 9221 by the Cancer and Leukemia Group B

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ABSTRACT

Purpose
Within the last two decades, a new understanding of the biology of myelodysplastic syndrome (MDS) has developed. With this understanding, new classification systems, such as the WHO diagnostic criteria, and the International Prognostic Scoring System and response criteria guidelines reported by the International Working Group (IWG) have been developed. We report the combined results of three previously reported clinical trials (n = 309) with azacitidine using the WHO classification system for MDS and acute myeloid leukemia (AML) and IWG criteria for response.

Patients and Methods
Data from three sequential Cancer and Leukemia Group B trials with azacitidine were recollected and reanalyzed as part of the New Drug Application process. The trials were conducted with either intravenous or subcutaneous azacitidine (75 mg/m^2/d for 7 days every 28 days).

Results
Complete remissions were seen in 10% to 17% of azacitidine-treated patients; partial remissions were rare; 23% to 36% of patients had hematologic improvement (HI). The median number of cycles to first response was three, and 90% of responses were seen by cycle 6. Using current WHO criteria, 103 patients had AML at baseline; 35% to 48% had HI or better responses. The median survival time for the 27 AML patients randomly assigned to azacitidine was 19.3 months compared with 12.9 months for the 25 patients assigned to observation. Furthermore, azacitidine did not increase the rate of infection or bleeding above the rate caused by underlying disease.

Conclusion
Azacitidine provides important clinical benefits for patients with high-risk MDS.

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INTRODUCTION

In 1984, the Cancer and Leukemia Group B (CALGB) began a series of clinical trials with azacitidine (Vidaza; Pharmion Corporation, Overland Park, KS) in patients with myelodysplastic syndrome (MDS).[1-4] These studies and other supportive data culminated in the 2004 US Food and Drug Administration approval of azacitidine for treatment of symptomatic patients with MDS. During the intervening two decades, a greater understanding of the biology of myelodysplasia has evolved, along with a new classification system developed by WHO that more clearly distinguishes MDS from acute myeloid leukemia (AML) and from chronic myeloproliferative disorders.[5,6] In addition, an International Working Group (IWG) sponsored by the National Cancer Institute (NCI) has published new response criteria for evaluation of new treatments for MDS.[7,8] As part of the New Drug Application process, Pharmion recollected and reanalyzed the CALGB data, including expert pathology review of blood and bone marrow slides. Some of the CALGB data from these three trials was previously published using the protocol-specified diagnostic and response criteria. Here, we report the combined results of a reanalysis using the WHO classification for MDS and AML and the IWG criteria for response in MDS.

PATIENTS AND METHODS

Data Collection

For the reanalysis, data were recollected from patients enrolled onto CALGB Protocols 8421, 8921, and 9221.[1-3] A comprehensive retrospective collection and reverification of all clinical data in the original protocols

were conducted. A complete safety database was collected, including additional data for azacitidine and data for the best supportive care (observation) arm of Protocol 9221; in the initial report by Silverman et al,[3] safety data were only collected and reported for azacitidine-treated patients. To validate the reliability of bone marrow slides and peripheral-blood films used for diagnosis and response, an independent blinded review was performed retrospectively (John M. Bennett) in addition to the previously conducted prospective local and central pathology reviews (Frederick R. Davey and Rose Ruth Ellison). All recollected data were entered into a new database held by Pharmion; our analyses were conducted separately from those reported in Silverman et al.[1-3]

Patient Selection

All patients enrolled onto CALGB Protocols 8421, 8921, and 9221 were included in our analyses. Consent forms were approved annually by local institutional review boards; each patient provided written consent. Detailed descriptions of the study designs of these three studies have been previously published.[1-3] Because Protocol 9221 included a comparative arm of best supportive care (observation), further analyses (eg, time to response, transfusion independence, and safety comparisons) were conducted.

Treatment Regimen

Although the route of administration was either continuous intravenous infusion (Protocol 8421) or subcutaneous (Protocols 8921 and 9221), the azacitidine dose investigated was 75 mg/m^2/d for 7 days, repeated in a 28-day cycle, in all three studies. No dose adjustments at inception of treatment were made for pre-existing cytopenias despite severity. Therapy was continued for three cycles after complete remission (CR) or until progressive disease or toxicity in patients with partial remission (PR) or hematologic improvement (HI). After 4 months, patients in the observation group could cross over to azacitidine after evidence of disease progression.[3]

Definitions

Treatment groups. In Protocol 9221, patients randomly assigned to azacitidine plus best supportive care were referred to as the azacitidine arm (n = 99); patients randomly assigned to best supportive care alone were referred to as the observation arm (n = 92). Patients randomly assigned to best supportive care alone but who did not cross over to azacitidine were referred to as the observation-only arm (n = 41); patients randomly assigned to best supportive care and who received azacitidine after cross over from the observation group were referred to as the azacitidine after observation arm (n = 51).

Response criteria. General differences between CALGB and IWG response criteria for MDS included duration of response (CALGB: ≥ 4 weeks; IWG: ≥ 8 weeks), normal target peripheral-blood values (CALGB: different criteria for hemoglobin targets for males and females; IWG: same criteria for males and females), and generally more stringent criteria for lineage response with the CALGB criteria. The IWG criteria for improvement differentiate major and minor responses by peripheral-blood values[7] but were combined in our analyses to give one overall calculation of improvement for patients.

WHO AML response rates. To evaluate responses in patients with MDS who were determined after expert pathology review to have AML at study entry, we applied the WHO classification for AML and removed refractory anemia with excess blasts (RAEB) in transformation (RAEB-T) from the MDS classification.[5,6] Therefore, our use of the WHO definition of AML includes patients previously defined as RAEB-T and AML by French-American-British criteria.

Transfusion independence. On the basis of IWG criteria, transfusion independence was defined as a transfusion-free period of ≥ 56 days (8 weeks) occurring anytime after random assignment. Transfusion dependence at baseline was defined as ≥ one transfusion within 90 days before random assignment.[8]

Adverse events. Any event occurring during the study not present at baseline or that worsened from baseline was documented as an adverse event (AE). All patients who received ≥ one dose of azacitidine were included in the azacitidine group as part of the safety analyses. For observation patients who crossed over, AEs that occurred during the period of observation were included in the observation safety analyses; any event that occurred after exposure to azacitidine was considered an azacitidine event and included in the azacitidine safety analyses.

Statistical Methods

For response rate analysis, the azacitidine group was compared with the observation-only group. For the survival analysis of patients with WHO AML, patients were analyzed as randomly assigned. If a patient was randomly assigned to the observation group, then all time to event data for that patient were analyzed in the observation group even if the patient later crossed over to azacitidine treatment. Safety analyses were performed on an as-treated basis.

Because the studies were conducted before availability of the IWG standardized response criteria for MDS, we retrospectively applied the IWG response criteria[7] for CR, PR, and HI. Time to response was measured from the date of random assignment (Protocol 9221) or entry onto the phase II studies (Protocols 8421 and 8921) to the date of the event.

The original 9221 study was not powered for overall survival in the subgroup of WHO AML patients. However, overall survival times were compared using the exact log-rank test. Statistical comparisons of groups in the proportion of patients achieving transfusion independence were performed using Fisher's exact test.

Numeric laboratory values were defined by a specific NCI Common Toxicity Criteria (CTC) grade, ranging from 0 to 4. The maximum NCI CTC grade within a cycle was used to represent that cycle. Baseline was determined as the maximum grade before the date of random assignment or, if no value was available before random assignment, as the earliest value after random assignment up to and including the day of the first dose of azacitidine for azacitidine patients, and baseline was determined as the maximum grade on the day of random assignment for observation patients.

To determine whether azacitidine was associated with an increase in either infection or bleeding, we examined the rates (in patient-years) of infection or bleeding AEs associated with azacitidine therapy compared with the rates seen with the underlying disease (on the observation arm). Summaries are inclusive of nonserious and serious AEs.

RESULTS

Patient Characteristics in Protocols 8421, 8921, and 9221

Among the three studies, 268 patients were treated with azacitidine, of whom 220 patients were treated with subcutaneous azacitidine and 48 were treated with intravenous azacitidine; 41 patients received best supportive care on the observation arm of Protocol 9221 (Table 1). The majority of all 309 patients registered or randomly assigned to azacitidine or observation were male (68%, 210 of 309 patients), white (94%, 292 of 309 patients), and ≥ 65 years of age (61%, 188 of 309 patients). The distribution of MDS subtypes at baseline diagnosis was similar among the azacitidine and observation groups in Protocol 9221. The percentage of patients with AML using WHO criteria was 52% in Protocol 8421, 37% in Protocol 8921, and 27% in the azacitidine arm and 27% (25 of 92 patients) in the observation arm of Protocol 9221.

Response in Protocols 8421, 8921, and 9221 Based on IWG Response Criteria for MDS

After applying the IWG response criteria to Protocols 8421, 8921, and 9221, response rates in the azacitidine groups were consistent across the studies, with between 40% and 47% of patients demonstrating a response. Ten percent to 17% of patients achieved a CR; PR was rare; and 23% to 36% of patients had HI (Table 2). Median duration of response was 13.1 months (range, 2 to 165.8+ months).

Patients With WHO AML

Because the previously discussed IWG response rates included some patients with an adjudicated diagnosis of AML, these patients were reviewed separately after redefining the AML diagnosis based on

Table 1. Baseline Demographics and Disease Characteristics in Protocols 8421, 8921, and 9221

Demographic	Protocol 8421: IV Azacitidine (n = 48)		Protocol 8921: SC Azacitidine (n = 70)*		Protocol 9221					
					SC Azacitidine† (n = 99)		Observation Only‡ (n = 41)		SC Azacitidine After Observation (n = 51)	
	No. of Patients	%	No. of Patients	%	No. of Patients	%	No. of Patients	%	No. of Patients	%
Sex										
Male	31	65	47	67	72	73	29	71	31	61
Female	17	35	23	33	27	27	12	29	20	39
Race										
White	48	100	66	94	93	94	38	93	47	92
Black	0	0	2	3	1	1	0	0	1	2
Hispanic	0	0	1	1	3	3	3	7	2	4
Asian	0	0	0	0	2	2	0	0	1	2
Other	0	0	1	1	0	0	0	0	0	0
Age, years										
No. of Patients	48		66		99		41		51	
Median	65.0		66.0		69.0		68.5		66.0	
Range	35-81		23-82		31-92		35-88		46-82	
Missing	0	0	4	6	0	0	1	2	0	0
< 65	21	44	26	37	36	36	16	39	17	33
65-74	24	50	24	34	39	39	11	27	22	43
≥ 75	3	6	16	23	24	24	13	32	12	24
Diagnosis at study entry§										
RA	0	0	7	10	16	16	3	7	17	33
RARS	0	0	4	6	6	6	2	5	5	10
RAEB	23	48	19	27	38	38	18	44	14	27
RAEB-T	24	50	16	23	17	17	7	17	9	18
CMMoL	0	0	14	19	12	12	6	15	2	4
AML	1	2	10	19	10	10	5	12	4	8
WHO AML‖	25	52	26	37	27	27	12	29	13	26
Performance status										
0, normal	15	31	19	27	35	35	13	32	13	26
1, fatigue	13	27	33	47	34	34	15	37	24	47
2, impaired	7	15	5	7	8	8	4	10	2	4
3, bed rest	3	6	0	0	1	1	0	0	0	0
Unknown/not performed	10	21	13	19	21	21	9	22	12	24
Transfusion product used within 3 months before study entry										
Any transfusion product	43	90	52	74	70	71	23	56	36	71
RBCs, packed	41	85	51	73	66	67	21	51	34	67
Hetastarch	0	0	0	0	0	0	0	0	1	2
Plasma protein fraction, human	0	0	0	0	1	1	0	0	0	0
Platelets,	16	33	20	29	15	15	7	17	5	10
Unknown	3	6	0	0	2	2	1	2	1	2

Abbreviations: IV, intravenous; SC, subcutaneous; RA, refractory anemia; RARS, refractory anemia with ringed sideroblasts; RAEB, refractory anemia with excess blasts; RAEB-T, refractory anemia with excess blasts in transformation; CMMoL, chronic myelomonocytic leukemia; AML, acute myeloid leukemia; WHO AML, WHO classification of AML.

*Two additional patients were registered (n = 72) but never received study medication.

†Patients randomly assigned to azacitidine.

‡Patients randomly assigned to observation who did not cross over to azacitidine.

§No patients were enrolled with a diagnosis of RA, RARS, or CMMoL in Protocol 8421 because this study only allowed patients with RAEB or RAEB-T myelodysplastic syndrome subtypes for enrollment.

‖Includes patients with RAEB-T and AML diagnosis after central review.

WHO criteria. Between 35% and 48% of patients in the azacitidine groups with WHO AML in Protocols 8421, 8921, or 9221 experienced CR, PR, or HI (Table 3). Among the 33 WHO AML responders in the three studies, the median duration of response was 7.3 months (range, 2.2 to 25.9 months).

In Protocol 9221, 7% of patients with WHO AML in the azacitidine group achieved CR or PR compared with 0% in the observation-only group. Median survival time for the 27 WHO AML patients in the azacitidine group was 19.3 months compared with 12.9 months for the 25 WHO AML patients randomly assigned to observation. Of 13 patients with WHO AML at study entry who crossed over to azacitidine, one patient who was on the observation arm for 5.2 months achieved PR, and one patient who was on the observation arm for 4.1 months achieved HI. Of the 11 patients who

Table 2. Best Response for All Patients Using IWG Response Criteria for MDS in Protocols 8421, 8921, and 9221

	Protocol 8421: IV Azacitidine (n = 48)		Protocol 8921: SC Azacitidine (n = 70)		Protocol 9221						Protocols 8921 and 9221: SC Azacitidine (n = 169)	
					SC Azacitidine* (n = 99)		Observation Only† (n = 41)		SC Azacitidine After Observation (n = 51)			
IWG Response	No. of Patients	%	No. of Patients	%	No. of Patients	%	No. of Patients	%	No. of Patients	%	No. of Patients	%
CR	7	15	12	17	10	10	0	0	3	6	22	13
PR	1	2	0	0	1	1	0	0	2	4	1	1
HI‡	13	27	16	23	36	36	7	17	13	25	52	31
Erythroid response, major	10	21	11	16	22	22	1	2	8	16	33	20
Erythroid response, minor	2	4	3	4	8	8	4	10	4	8	11	7
Platelet response, major	9	19	6	9	21	21	2	5	3	6	27	16
Platelet response, minor	0	0	2	3	3	3	0	0	1	2	5	3
Neutrophil response, major	2	4	0	0	8	8	1	2	2	4	8	5
Neutrophil response, minor	0	0	0	0	0	0	0	0	0	0	0	0
Overall: CR + PR + HI‡	21	44	28	40	47	47	7	17	18	35	75	44

Abbreviations: IWG, International Working Group; MDS, myelodysplastic syndrome; IV, intravenous; SC, subcutaneous; CR, complete remission; PR, partial remission; HI, hematologic improvement.
*Patients randomly assigned to azacitidine.
†Patients randomly assigned to observation who did not cross over to azacitidine.
‡Patients with HI (major or minor) were counted only once in the overall response.

crossed over to azacitidine but had no response, the median time to cross over was 3.3 months after study entry (range, 1.4 to 10.3 months). Among WHO AML patients who were transfusion independent at baseline, duration of transfusion independence was significantly longer for patients in the azacitidine group compared with the observation group for both RBC (azacitidine: 14.7 months, n = 8; observation: 4.8 months, n = 9; $P = .02$) and platelets (azacitidine: 13 months, n = 13; observation: 4.5 months, n = 18; $P = .004$).

Time to Response and Duration of Response Based on IWG Response Criteria in Protocols 8421, 8921, and 9221

In all three studies, the median number of cycles from the first treatment with azacitidine to any response (CR, PR, or HI) was three cycles (range, one to 17 cycles). Although 75% of the responders achieved a response by cycle 4, the other 25% achieved a response as late as cycle 17. The majority of responders (90%) achieved a response

Table 3. Best Response for All Patients With AML (WHO) in Protocols 8421, 8921, and 9221 Using IWG Response Criteria for MDS

	Protocol 8421: IV Azacitidine (n = 25)		Protocol 8921: SC Azacitidine (n = 26)		Protocol 9221						Protocols 8921 and 9221: SC Azacitidine (n = 53)	
					SC Azacitidine* (n = 27)		Observation Only† (n = 12)		SC Azacitidine After Observation (n = 13)			
IWG Response	No. of Patients	%	No. of Patients	%	No. of Patients	%	No. of Patients	%	No. of Patients	%	No. of Patients	%
CR	3	12	3	12	2	7	0	0	0	0	5	9
PR	1	4	0	0	0	0	0	0	1	8	0	0
HI‡	8	32	6	23	8	30	0	0	1	8	14	26
Erythroid response, major	5	20	4	15	6	22	0	0	1	8	10	19
Erythroid response, minor	1	4	1	4	0	0	0	0	0	0	1	2
Platelet response, major	5	20	1	4	6	22	0	0	0	0	7	13
Platelet response, minor	0	0	1	4	1	4	0	0	0	0	2	4
Neutrophil response, major	0	0	0	0	1	4	0	0	1	8	1	2
Neutrophil response, minor	0	0	0	0	0	0	0	0	0	0	0	0
Overall: CR + PR + HI‡	12	48	9	35	10	37	0	0	2	15	19	36

Abbreviations: AML, acute myeloid leukemia; IWG, International Working Group; MDS, myelodysplastic syndrome; IV, intravenous; SC, subcutaneous; CR, complete remission; PR, partial remission; HI, hematologic improvement.
*Patients randomly assigned to azacitidine.
†Patients randomly assigned to observation who did not cross over to azacitidine.
‡Patients with HI (major or minor) were counted only once in the overall response.

Table 4. Proportion and Duration of RBC Transfusion-Free* Periods in Responders Who Were Previously Transfusion-Dependent Patients in Protocol 9221

Treatment and FAB Classification†	No. of Patients	Patients Independent After Random Assignment		Duration of Transfusion Independence (months)	
		No.	%	Median	Range
RBC transfusion-dependent patients at baseline‡ who achieved CR, PR, or HI	45	36	80		
Azacitidine	29	25	86	9.0	2.0-63.0
RA	6	6	100	13.0	2.0-63.0
RARS	2	1	50	17.3	
RAEB	10	9	90	8.6	2.5-33.4
RAEB-T	3	3	100	9.4	6.8-10.3
CMMoL	5	3	60	8.4	4.0-10.0
AML	3	3	100	10.9	3.8-25.8
Observation only	5	1	20	2.3	—
RA	0	0	0		
RARS	0	0	0		
RAEB	3	1	33	2.3	—
RAEB-T	0	0	0		
CMMoL	2	0	0		
AML	0	0	0		
Azacitidine after observation	11	10	91	9.6	2.0-88.4
RA	3	3	100	6.7	5.3-14.8
RARS	1	1	100	12.4	—
RAEB	5	5	100	6.6	3.0-88.4
RAEB-T	2	1	50	12.9	—
CMMoL	0	0	0		
AML	0	0	0		

NOTE. Comparison of proportion of patients achieving a transfusion-free period using Fisher's two-sided exact test: azacitidine *v* observation only, $P = .007$.
Abbreviations: FAB, French-American-British; CR, complete remission; PR, partial remission; HI, hematologic improvement; RA, refractory anemia; RARS, refractory anemia with ringed sideroblasts; RAEB, refractory anemia with excess blasts; RAEB-T, refractory anemia with excess blasts in transformation; CMMoL, chronic myelomonocytic leukemia; AML, acute myeloid leukemia.
*Transfusion-free period defined as ≥ 56 days.
†On the basis of adjudicated review at baseline and those patients with blasts > 20%, the No. of WHO AML patients included six in the azacitidine group, zero in the observation group, and two in the azacitidine after observation group.
‡Transfusion administered within 90 days before random assignment.

by cycle 6. Best response was observed, on average, two cycles after initial response and lasted for a median of five cycles.

Transfusion Independence in Protocol 9221

Of the 191 patients enrolled onto Protocol 9221, 121 (63%) were RBC transfusion dependent at baseline. Of these RBC transfusion–dependent patients, 45 showed a response (CR, PR, or HI). A total of 36 of the responders became transfusion independent, of whom 35 had received azacitidine (Table 4). Among all RBC transfusion–dependent patients treated initially with azacitidine, 45% (29 of 65 patients) achieved RBC transfusion independence. A similar trend was noted for the 27 patients who were platelet transfusion dependent at baseline. Furthermore, among all 72 overall responders, platelet transfusion independence was achieved in all eight patients randomly assigned to azacitidine and in both patients who received azacitidine after cross over.

The median duration of RBC transfusion independence was 9 months in patients with CR, PR, or HI who were transfusion dependent before receiving azacitidine (n = 25) compared with 2.3 months for observation-only patients (n = 1; Table 4). A similar trend was noted for platelet transfusion independence, for which the median duration of independence was 6.3 months in the azacitidine group (n = 8) compared with 2.4 months for the observation-only group (n = 1).

Safety Results in Protocol 9221

Hematology. Mean times to nadir values (across all cycles) for RBC, hemoglobin, WBC, absolute neutrophils, and platelets were between 15 and 16 days. On the basis of the overall frequency of time to nadir within the cycle (days 1 to 7, 8 to 14, 15 to 21, or > 21), hematology values tended to reach nadir values during the second or third week of the cycle (Table 5).

Most patients had NCI CTC grade 0 to 2 hematology values at baseline (Table 6). Many patients in both treatment groups had shifts

Table 5. Onset of Hematology Nadir Values in Cycle Day (all cycles combined) in Protocol 9221 for All Azacitidine Patients (N = 150)

Hematology Value	No. of Patients	Cycle Day of Onset of Nadir Values		
		25th Percentile	Median	75th Percentile
Hemoglobin	149	11	15	19
WBCs	149	13	16	20
Absolute neutrophils	143	12	17	22
Platelets	148	13	15	18

NOTE. Table includes all patients exposed to azacitidine, including patients who crossed over to azacitidine from observation.

Table 6. Summary of Hematology Shifts by Maximum NCI CTC Grade Criteria (hematology) in Protocol 9221

Maximum NCI CTC Grade Baseline Value	Postbaseline* Maximum NCI CTC Grade: All Azacitidine Patients† (n = 150) No. of Patients	Grades 0-2 No.	Grades 0-2 %	Grade 3 No.	Grade 3 %	Grade 4 No.	Grade 4 %	Observation (n = 92) No. of Patients	Grades 0-2 No.	Grades 0-2 %	Grade 3 No.	Grade 3 %	Grade 4 No.	Grade 4 %
Hemoglobin														
Grades 0-2	105	27	26	62	59	16	15	61	33	54	19	31	9	15
Grade 3	29	2	7	17	59	10	35	14	3	21	5	36	6	43
Grade 4	9	0	0	3	33	6	67	6	0	0	3	50	3	50
WBCs														
Grades 0-2	117	49	42	47	40	21	18	71	61	86	8	11	2	3
Grade 3	27	0	0	9	33	18	67	15	2	13	11	73	2	13
Grade 4	5	0	0	0	0	5	100	3	0	0	0	0	3	100
Absolute neutrophil count														
Grades 0-2	66	12	18	11	17	43	65	36	27	75	6	17	3	8
Grade 3	22	2	9	0	0	20	91	12	2	17	5	42	5	42
Grade 4	23	2	9	1	4	20	87	9	1	11	0	0	8	89
Lymphocytes														
Grades 0-2	71	23	32	31	44	17	24	38	27	71	9	24	2	5
Grade 3	28	0	0	13	46	15	54	19	2	11	11	58	6	32
Grade 4	9	1	11	2	22	6	67	9	1	11	3	33	5	56
Platelets														
Grades 0-2	80	34	43	19	24	27	34	53	38	72	11	21	4	8
Grade 3	34	0	0	7	21	27	79	18	0	0	4	22	14	78
Grade 4	24	0	0	0	0	24	100	12	0	0	0	0	12	100

NOTE. The No. (n) for each treatment group is the No. of patients with a baseline toxicity grade and at least one postbaseline toxicity grade.
Abbreviation: NCI CTC, National Cancer Institute Common Toxicity Criteria.
*Postbaseline toxicity grade is the maximum grade after baseline and before the end of study.
†Includes all patients exposed to azacitidine, including patients who crossed over to azacitidine from observation.

from grade 0 to 2 at baseline to grade 3 to 4. Azacitidine was associated with worsening of pre-existing cytopenias in ≤ 78% of patients in Protocol 9221. In general, the percentage of patients with shifts from grade 0 to 2 to grade 4 in hematology values was greatest during cycle 1 and then decreased with subsequent cycles.

AEs. The overall rate of AEs per patient-year in the observation group (2.06 patients with events per patient-year of exposure) was nearly twice the rate in the azacitidine group (1.09 patients with events per patient-year of exposure; Table 7). Of the most frequently observed AEs, GI events and injection site reactions occurred at a greater rate in the azacitidine group compared with the observation group. Hematologic events characteristic of MDS generally occurred at a greater rate in the observation group.

Rates of infection and bleeding. To determine whether treatment was associated with an increase in either infection or bleeding, we examined the rates of commonly reported AEs of infection or bleeding. Infections occurred in the observation group at nearly 1 per patient-year, which is similar to the rate previously reported.[9] Treatment with azacitidine did not increase the rate of infection. The rate of infection per patient-year was 0.64 in the azacitidine group and 0.95 in the observation group. Clinically significant infections were similar to the most common sites of infection (lung, urinary tract, and the bloodstream) typically observed in patients with MDS,[9] with no apparent increase in the azacitidine group (Table 8). In the observation group, infection with pneumonia/sepsis was the cause of death in month 3 of one (2%) of the 41 observation patients who did not cross over during the study. Among 150 azacitidine-treated patients, infection (pneumonia, cycle 68; infection, cycle 4; and probable infection, cycle 2) was the cause of death in three patients (2%).

There was no increase in rates of bleeding with azacitidine. The overall rate (patient-years) of bleeding was 0.56 in the azacitidine group and 0.60 in the observation group. Clinically relevant bleeding in the GI, CNS, renal, and pulmonary systems seemed to occur at a similar rate (Table 9). The incidence of bleeding leading to death seemed similar between the observation (2%; one of 41 patients) and azacitidine groups (1%; two of 150 patients). Events leading to death included a subdural hematoma (cycle 5) in the observation group and two episodes of intracranial hemorrhage (cycles 1 and 5) in the azacitidine group.

DISCUSSION

The findings of this study, which are based on more contemporary classification and response criteria, validate the previously published results[1-3] and further our understanding of the activity of azacitidine in the treatment of MDS. Furthermore, these results provide additional findings based on a thorough recollection of data in a manner consistent with Good Clinical Practice, which included a complete safety database, and provide further support for the significant improvement in the quality of life of patients treated with azacitidine reported in a CALGB companion study.[4]

When the IWG response criteria were applied to all three studies, overall response rates were generally consistent among the studies and

Table 7. Most Frequently* Observed Adverse Events (NCI CTC grades 1-4) by Patient-Years of Exposure in Protocol 9221

Adverse Event‡	All Azacitidine Patients† (n = 150)		Observation Patients (n = 92)	
	Patients With Events Per Patient-Year of Exposure§	No. of Patients	Patients With Events Per Patient-Year of Exposure§	No. of Patients
Total exposure, patient-years	138.2		43.2	
At least 1 adverse event	1.09	150	2.06	89
Anemia NOS	0.77	107	1.37	59
Thrombocytopenia	0.74	102	0.97	42
Nausea	0.72	100	0.37	16
Pyrexia	0.56	77	0.65	28
Leukopenia NOS	0.55	76	0.63	27
Vomiting NOS	0.52	72	0.12	5
Fatigue	0.42	58	0.53	23
Constipation	0.42	58	0.14	6
Diarrhea NOS	0.39	54	0.30	13
Neutropenia	0.37	51	0.23	10
Injection site erythema	0.35	49	0	0
Cough	0.34	47	0.32	14
Dyspnea NOS	0.34	47	0.25	11
Ecchymosis	0.32	44	0.32	14
Weakness	0.32	44	0.44	19
Rigors	0.28	39	0.23	10
Injection site pain	0.26	36	0	0
Arthralgia	0.26	36	0.07	3
Headache NOS	0.25	34	0.23	10
Pain in limb	0.25	34	0.12	5
Anorexia	0.23	32	0.14	6
Pharyngitis	0.23	32	0.16	7
Contusion	0.22	31	0.21	9

Abbreviations: NCI CTC, National Cancer Institute Common Toxicity Criteria; NOS, not otherwise specified.
*More than or equal to 20.0% frequency in either treatment group.
†Includes all patients exposed to azacitidine, including patients who crossed over to azacitidine from observation.
‡Multiple reports of the same adverse event term for a patient are only counted once within each treatment group.
§Total exposure for azacitidine is the cumulative time from the first dose to the end of study (30 days after last dose), and for observation, total exposure is the cumulative time from random assignment to withdrawal from study or day before cross over.

with the original CALGB response rates. However, the IWG criteria had lower sensitivity in determining PR rates when compared with the CALGB criteria. IWG criteria had lower sensitivity to discriminate responses, as demonstrated by 17% of patients in the observation-only group who qualified for an IWG response of HI (10% minor hematologic response) compared with only 5% of patients using the original CALGB criteria.

The overall IWG response rates for patients with a retrospective diagnosis of WHO AML were encouraging. Although the CR rate of 9% using IWG MDS response criteria is not outstanding

Table 8. NCI CTC Grades 1 to 4 Infection Rates (patient-years of exposure) in Protocol 9221

Adverse Event†	Azacitidine Patients* (n = 150)		Observation Patients (n = 92)	
	Patients With Events Per Patient-Year of Exposure‡	No. of Patients	Patients With Events Per Patient-Year of Exposure‡	No. of Patients
Total infections§	0.64	89	0.95	41
Candidal infection NOS	0.04	6	0.07	3
Cellulitis	0.09	13	0.09	4
Herpes simplex	0.09	13	0.12	5
Oral candidiasis	0.04	5	0.07	3
Pneumonia NOS	0.10	14	0.12	5
Sepsis NOS	0.04	6	0.12	5
Urinary tract infection NOS	0.09	12	0.12	5

Abbreviations: NCI CTC, National Cancer Institute Common Toxicity Criteria; NOS, not otherwise specified.
*Includes all patients exposed to azacitidine, including patients who crossed over to azacitidine from observation.
†Multiple reports of the same adverse event term for a patient are only counted once within each treatment group.
‡Total exposure for azacitidine is the cumulative time from the first dose to the end of study (30 days after last dose), and for observation, total exposure is the cumulative time from random assignment to withdrawal from study or day before cross over.
§From infections and infestations system organ class using MedDRA, version 5.0 (Northrop Grumman, Los Angeles, CA).

Table 9. NCI CTC Grades 1 to 4 Bleeding Rates (patient-years of exposure) in Protocol 9221

Adverse Event†	Azacitidine Patients* (n = 150)		Observation Patients (n = 92)	
	Patients With Events Per Patient-Year of Exposure‡	No. of Patients	Patients With Events Per Patient-Year of Exposure‡	No. of Patients
Total bleeding§	0.56	77	0.60	26
GI disorders§	0.26	36	0.25	11
GI hemorrhage	0.03	4	0	0
Gingival bleeding	0.13	18	0.09	4
Hemorrhoidal hemorrhage	0.04	6	0	0
Melena	0.03	4	0.05	2
Oral hemorrhage	0.04	5	0.02	1
Rectal hemorrhage	0.05	7	0.05	2
Total nervous system disorders§	0.01	2	0.02	1
Intracranial hemorrhage	0.01	2	0	0
Subdural hematoma	0	0	0.02	1
Total renal and urinary disorders,§ hematuria	0.05	7	0.07	3
Total respiratory, thoracic and mediastinal disorders§	0.22	30	0.23	10
Epistaxis	0.18	25	0.21	9
Hemoptysis	0.05	7	0.02	1

Abbreviations: NCI CTC, National Cancer Institute Common Toxicity Criteria; NOS, not otherwise specified.
*Includes all patients exposed to azacitidine, including patients who crossed over to azacitidine from observation.
†Multiple reports of the same adverse event term for a patient are only counted once within each treatment group.
‡Total exposure for azacitidine is the cumulative time from the first dose to the end of study (30 days after last dose), and for observation, total exposure is the cumulative time from random assignment to withdrawal from study or day before cross over.
§System organ class using MedDRA, version 5.0 (Northrop Grumman, Los Angeles, CA).

relative to standard AML remission induction chemotherapy, the prolongation in survival time to 19.3 months exceeds that typically seen with standard induction chemotherapy, suggesting that azacitidine may alter the natural history of the disease independent of CR response criteria.[10] Furthermore, treatment with azacitidine is associated with significant reduction in risk of transformation to AML and a significant prolongation of survival in patients with high-risk MDS, including RAEB patients, RAEB-T patients ≥ 65 years of age, and patients with equivalent intermediate-2 and high-risk disease.[11,12] This finding and the data presented in this article suggest a paradigm shift, with azacitidine altering the natural history of MDS by modulating the behavior of the MDS clone without necessarily eradicating it. In addition, treatment with azacitidine significantly delays the onset of RBC and platelet transfusions in patients who are transfusion independent at study entry. These findings warrant further studies of azacitidine in patients with smoldering AML with multilineage dysplasia (ie, patients who were previously diagnosed as having RAEB-T).

The time to response data indicate that azacitidine can have an effect at the bone marrow level as early as the first treatment cycle. However, for this effect to translate into an improvement in bone marrow function leading to clinically significant increases in peripheral cell counts, the majority of responders can require up to six cycles of azacitidine. To ensure adequate exposure for patients to demonstrate a clinical response, azacitidine should be administered for a minimum of four cycles. Furthermore, patients will most likely continue to require transfusion support during the first several cycles of treatment with azacitidine. In patients who were transfusion dependent at baseline with a response, azacitidine was associated with a median of 9 months of transfusion independence.

Given the underlying disease process and the myelotoxicity of compounds such as azacitidine and decitabine, an increase in rates of infection and bleeding would be expected during treatment. Despite the potential to exacerbate pre-existing cytopenias early in therapy, azacitidine did not increase the rate of infection or bleeding above the rate caused by underlying disease.

This reanalysis demonstrates that azacitidine is effective therapy that directly impacts the disease of MDS rather than just managing the symptoms. It reconfirms the findings discussed in Silverman et al[3] and adds additional data pertaining to safety and more current classification and response criteria. Furthermore, azacitidine warrants additional studies in patients with AML with dysplasia.

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Appendix

The Appendix is included in the full-text version of this article, available online at www.jco.org. It is not included in the PDF version (via Adobe® Reader®).

Authors' Disclosures of Potential Conflicts of Interest

Although all authors completed the disclosure declaration, the following authors or their immediate family members indicated a financial interest. No conflict exists for drugs or devices used in a study if they are not being evaluated as part of the investigation. 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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7089 General Poster Session (Board #43G), Sat, 8:00 AM - 12:00 PM

Relationship of progression to acute myeloid leukemia (AML) from myelodysplastic syndrome (MDS) and cytogenetic status. *E. Hellstrom-Lindberg, P. Fenaux, G. J. Mufti, A. F. List, V. Santini, S. Gore, J. F. Seymour, J. Backstrom, D. McKenzie, C. L. Beach; Hopital Avicenne, Universite Paris XIII, Bobigny, France; King's College Hospital, London, United Kingdom; H. Lee Moffitt Cancer Center, Tampa, FL; University of Florence, Florence, Italy; Sidney Kimmel Comprehensive Cancer Center, Baltimore, MD; Peter MacCallum Cancer Centre, East Melbourne, Australia; Pharmion Corporation, Overland Park, KS*

Background: A recent phase III trial showed that azacitidine (AZA) significantly prolonged overall survival in pts with high-risk MDS, regardless of a good, intermediate, or poor IPSS karyotype (Fenaux, Blood 2007;110,817). Using cytogenetic data from this trial, our subgroup analysis assessed changes in baseline (BL) cytogenetic status in MDS pts who subsequently transformed to AML (>30% bone marrow blasts). **Methods:** In all, 358 pts met inclusion criteria of high-risk MDS, defined as RAEB, RAEB-T, or CMMoL per FAB with an IPSS of Int-2 or High. Pts were randomized to AZA (75 mg/m^2/d x 7d, q 28d) or CCR with both groups receiving concomitant best supportive care (BSC: transfusions, antibiotics, and G-CSF only for neutropenic infection) as needed. CCR consisted of 3 treatments: BSC only, low-dose ara-C (20 mg/m^2/d x 14d, q28d), or standard chemotherapy (7+3 regimen, induction/consolidation). No erythropoietin was allowed. **Results:** For this subgroup analysis, complete cytogenetic data were available from 78 (40 AZA; 38 CCR) of the 358 pts. Baseline characteristics were similar across the 2 arms (70% male; median age, 69 years). In all, 92% of pts had RAEB/RAEB-T disease; 87% an IPSS of Int-2 or High. Median time to transformation to AML during the treatment period was 26.1 months and 12.4 months in the AZA and CCR groups (p=0.004), respectively; 33% of AZA pts versus 31% of CCR pts transformed. In either arm, the IPSS cytogenetic status (good, intermediate, or poor) of the majority of pts remained unchanged from BL to transformation to AML (Table). **Conclusion:** These data demonstrate that transformation to AML in high-risk pts is rarely accompanied by a worsening in IPSS cytogenetic status and that treatment with AZA is associated with a significant delay in transformation.

BL Cytogenetic Status	Cytogenetic Status at AML Transformation						AZA	CCR
	AZA			CCR				
	Good	Int	Poor	Good	Int	Poor		
Good	20	20	20	0	0	18	2	0
Int	7	9	0	6	1	1	8	0
Poor	10	7	1	0	9	0	0	7

7090 General Poster Session (Board #43H), Sat, 8:00 AM - 12:00 PM

Use of single nucleotide polymorphism (SNP) array karyotyping to detect clonal chromosomal abnormalities in myelodysplastic syndrome (MDS) and refractory anemia with ringed sideroblasts (RARS). *T. Ghazal, A. S. Haddad, L. P. Gondek, K. S. Theil, M. A. Sekeres, A. Lichtin, J. P. Maciejewski; Cleveland Clinic, Cleveland, OH*

Background: The current standard for identification of chromosomal abnormalities in MDS is metaphase cytogenetics (MC). However, this technique has low resolution and requires bone marrow sampling and cell growth in vitro. High density SNP arrays offer significantly higher resolution for identification of previously cryptic chromosomal (chr.) abnormalities. This study aims to elucidate the accuracy of SNP arrays compared to MC, and to measure concordance between testing on peripheral blood (PB) and bone marrow (BM) tissues. **Methods:** DNA was available for 38 pts (PB = 15, BM = 23) with ringed sideroblasts (RS) and was subjected to 250K SNP-A karyotyping. Pathologic lesions were defined upon exclusion of normal copy number polymorphisms identified in 81 controls, as well as the Database of Genomic Variants. **Results:** By MC, a defective karyotype was present in 17/38 pts (44%). When SNP-A was applied as a karyotyping tool (copy number and loss of heterozygosity (LOH) analysis), all aberrations found by MC were confirmed but new lesions were also detected so that an abnormal karyotype was established in 63% of patients (pts). Several previously cryptic lesions included losses of a portion of chr. 17 (N=3; 17q21.31, 17q21.33, 17q11.2) as well as gains (N=2; 17q12, 17q21.31). We have also detected segmental uniparental disomy (UPD) in chr 1 (N=3; 1p21.3–22.2, 1p, 1p35.1-pter). This type of lesion cannot be detectec using MC and provides an additional mechanism leading to LOH. When both bone marrow and blood of 5 RARS pts were tested using SNP-A, blood analysis had 100% accuracy rate as compared to marrow. All defects seen in the marrow were also found in blood. **Conclusions:** Chromosomal defects are present in RARS patients and that arrays with higher resolution will detect more defects than MC. Our study also demonstrates testing of peripheral blood by SNP-A can complement marrow MC, especially in cases in which marrow is not available. Detection of clonal marker aberrations in blood of RARS patients suggests that mostly clonal dysplastic progenitor cells contribute to blood production rather than residual "normal" progenitors.

7091 General Poster Session (Board #44A), Sat, 8:00 AM - 12:00 PM

A phase I, open-label, dose-escalation study to evaluate the safety, pharmacokinetics, and pharmacodynamics of oral azacitidine in subjects with myelodysplastic syndromes (MDS) or acute myelogenous leukemia (AML). *B. S. Skikne, M. R. Ward, A. Nasser, L. Aukerman, G. Garcia-Manero; University of Kansas Medical Center, Kansas City, KS; Pharmion Corporation, Boulder, CO; University of Texas M. D. Anderson Cancer Center, Houston, TX*

Background: Azacitidine, a cytidine analog, through its effects on DNA metabolism, gene expression, and cell differentiation, has proven beneficial in treatment of MDS and AML. Most notably, prolonged azacitidine therapy recently has been shown to approximately double 2-year survival in higher-risk MDS subjects compared to conventional care. Azacitidine is currently approved for intermittent subcutaneous (SC) and intravenous administration. Development of an oral formulation would provide more convenient dosing, eliminate injection-site reactions, and allow evaluation of novel, continuous low-dose regimens that may sustain demethylation and improve efficacy. A proprietary formulation of oral azacitidine was shown to be absorbed in a pilot, single-dose study (ASCO 2007). **Methods:** This is a multicenter, open-label, Phase 1, sequential design, dose-escalation study of oral azacitidine. The study is designed to evaluate the maximum tolerated dose (MTD), dose limiting toxicities (DLTs), safety, pharmacokinetic (PK) and pharmacodynamic (PD) profiles of increasing doses of orally administered azacitidine in subjects with MDS or AML. Azacitidine was administered SC (75 mg/m^2/day x 7 days) during cycle 1, then orally starting at 120 mg x 7 days/28 day cycle. Drug levels were measured in plasma and urine, and PD effects including global LINE methylation and gene-specific methylation were assayed. **Results:** Currently, no toxicities have been observed in subjects who have completed both the SC and oral phases of the study at 120 mg. The study continues at the 180 mg dose level. Preliminary PK analysis indicates detectable plasma levels at the 120 mg oral dose. A comparison of plasma PK profiles and PD effects of azacitidine administered at increasing oral doses compared to those of azacitidine administered SC at the approved dose of 75 mg/m^2/day for all subjects evaluated to date will be presented. **Conclusions:** Initial results of oral 5-azacitidine indicate that this formulation is orally bioavailable and safe in subjects with MDS.

7092 General Poster Session (Board #44B), Sat, 8:00 AM - 12:00 PM

Lack of correlation of eculizumab therapy in paroxysmal nocturnal hemoglobinuria (PNH) patients with myeloproliferative disorders, myelodysplastic syndromes, acute leukemias or aplastic anemia with long-term treatment. *J. Schubert, N. S. Young MD, L. Luzzatto, R. A. Brodsky, G. Socié, R P. Rother, S. A. Rollins, P. Hillmen; Saarland University Medical School, Homburg, Saar, Germany; National Heart, Lung, and Blood Institute, Bethesda, MD; Istituto Toscano Tumori, Florence, Italy; Johns Hopkins University School of Medicine, Baltimore, MD; Hospital Saint Louis and INSERM, Paris, France; Alexion Pharmaceuticals, Cheshire, CT; St. James' Institute of Oncology, Leeds, United Kingdom*

Background: PNH is a rare disorder characterized by bone marrow dysfunction and expansion of stem cell clones with somatic mutations in the PIG-A gene. Mutations in PIG-A result in deficiency of GPI-anchored proteins on the surface of blood cells including the complement inhibitor CD59. Lack of CD59 from PNH RBCs results in chronic hemolysis and leads to fatigue, thrombosis, anemia and poor quality of life. Development of myeloproliferative disorders, MDS, acute leukemias and AA/pancytopenia has been reported in PNH. In a previous retrospective analysis, 6.8% of patients developed either myeloid or lymphoid disorders and 1% developed acute leukemias. AA/pancytopenia has been reported to develop in PNH patients at a rate of ~2%/year. Eculizumab prevents terminal complement activation and restores the PNH RBC clone resulting in improvements in anemia, thrombosis, fatigue, and quality of life. Eculizumab has been evaluated in 195 PNH patients initially enrolled in 1 of 3 parent trials who continued to receive drug in an extension trial (>382 patient years of exposure). **Methods:** Cell counts for RBCs, WBCs, platelets and neutrophils and leukocyte PNH clone size were assessed at baseline and at 18 mos. The frequency of MDS, leukemia and AA/pancytopenia was calculated across all eculizumab trials. **Results:** RBC, WBC, platelet and neutrophil counts did not change during 18 mos of eculizumab. PNH leukocyte clone size did not increase with eculizumab (median 96.7% at baseline vs 96.7% at 18 mos) suggesting that inhibition of complement does not destroy or result in the proliferation of PNH leukocyte clones. Additionally, the development of MDS (1/195 patients, 0.5%) and CMML (1/195 patients, 0.5%) during the treatment period was not increased over that expected. The case of CMML was attributed to a previously diagnosed high grade myelodysplasia. Interestingly, no new AA/pancytopenia cases developed during eculizumab, while 7–8 cases would have been expected from the reported rate. **Conclusions:** Long-term terminal complement inhibition with eculizumab does not increase myeloproliferative disorders, MDS, acute leukemias or AA/pancytopenia in PNH patients.

Am J Clin Oncol (CCT) 5: 411–415, 1982.

Phase II study of 5-azacytidine in sarcomas of bone

Usha Srinivasan
Gregory H. Reaman*
David G. Poplack
Daniel L. Glaubiger
Arthur S. Levine

EIGHT PATIENTS WITH OSTEOGENIC SARCOMA and seven patients with Ewing's sarcoma, all with advanced metastatic disease refractory to conventional therapy, were treated with 5-azacytidine (NSC-102816) intravenously at a dosage of 150 mg/m² in three divided doses daily × 5 days. The courses were repeated q 4 weeks and the dosage was escalated to 200 mg/m²/day, as tolerated. Fourteen patients were evaluable for response. Major toxicities were hematologic and gastrointestinal. 5-azacytidine had no demonstrable antitumor activity.

5-Azacytidine is a pyrimidine analogue of cytidine with a nitrogen atom substituted for carbon in the number 5 position of the ring. This antimetabolite was synthesized by Piskala and Sorm in 1964,[8] and was subsequently isolated from a culture of streptoverticillium ladakanus in 1966.[5] The drug acts as an analogue of cytidine and affects the synthesis of both DNA and RNA.[7] An extensive bibliography of the investigations on the mechanism of action of this drug is included in a review by Carter and Slavik.[2]

Previous studies have shown that 5-azacytidine has significant antitumor activity in murine L1210 leukemia and T-4 lymphoma[5,7] and human acute nonlymphoblastic leukemia.[6,10] Activity against solid tumors by this agent was demonstrated in a phase I study, with objective remissions occurring in patients with breast and colon cancer and malignant melanoma.[13] This report details a phase II evaluation of 5-azacytidine in patients with osteogenic sarcoma and Ewing's sarcoma refractory to conventional therapy.

Materials and methods

Patients with histologically proven osteogenic sarcoma or Ewing's sarcoma with advanced measurable disease, who had failed conventional therapy in phase III clinical trials and had a life expectancy of at least 8 weeks, were eligible for this study. A total of 15 such patients were entered on protocol. Informed consent was obtained from the patient and/or parents prior to starting treatment with 5-azacytidine. The pre-treatment evaluation

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consisted of a complete history and physical examination, CBC, platelet count, x-rays of primary and metastatic sites, a Tc^{99} bone scan, and serum chemistries (including electrolytes, BUN, creatinine, uric acid, transaminases, bilirubin, LDH, alkaline phosphatase, total protein, albumin, calcium, phosphorus and magnesium). Computerized axial tomography (CT), whole lung tomography and gallium scans were obtained as indicated. CBC and platelet counts were repeated every other day during the 5-day course, and weekly between courses. Serum chemistries were determined, and x-rays of primary and known metastatic sites were done at the completion of, and prior to, every course. Radionuclide and CT scans were repeated as required to assess measurable disease.

Treatment plan

5-Azacytidine (NSC-102816) was supplied as a white lyophilized powder, 100 mg in 20 ml vials. This was reconstituted with sterile water for injection, diluted in Ringer's lactate, and given as an intravenous infusion over a 3-hour period immediately after reconstitution. The outline of the treatment regimen is shown in Table 1. The dosage in the first course was 150 mg/m^2/day, given at 8-hour intervals on days 1–5. If this was tolerated, the dosage was escalated to 200 mg/m^2/day in the second course. In the presence of serious toxicity, the dosage was limited to 150 mg/m^2/day. Serious toxicity was defined as neutropenia (granulocyte count <500/mm^3) and/or thrombocytopenia (platelet count <20,000/mm^3) for >30 days, severe hepatotoxicity (see below) or incapacitating neuromuscular toxicity. The rest period between courses was for a minimum of 21 days, and until the absolute granulocyte count was >1000/mm^3.

Criteria for response

A complete response was defined as complete disappearance of radiographically measurable lesions. A partial response was defined as at least a 50% reduction in size of all measurable tumor areas and these parameters had to be present for at least 2 measurement periods separated by at least 3 weeks.

Grading of toxicity

Toxicity encountered during therapy was graded as follows: neutrophil count—grade 1 = 500–1000/mm^3, and grade 2 = <500/mm^3, platelet count—grade 1 = 50,000–100,000/mm^3, grade 2 = 20,000–50,000/mm^3, and grade 3 = <20,000/mm^3; hemoglobin level—grade 1 = a fall of 2–4 grams/100 ml, and grade 2 = a fall of >4

TABLE 1

Treatment Plan

5-Azacytidine 150 mg/m^2/day ÷ 3 doses I.V. days 1–5 (3 hr. infusion 1 8 hrs.)

Rest period[a]

Evidence of regression of lesions + no significant toxicity	Evidence of regression or stabilization of lesions + significant toxicity[b]	Evidence of progression of lesions
to 200 mg/m^2/day X 5 days	Continue at 150 mg/m^2/day X 5 days	Off study
Rest period[a]	Rest period[a]	Other therapy
Continue at maximally tolerated dose (maximum: 200 mg/m^2/day)	Off study if (a) serious toxicity prevents further therapy with 5-azacytidine or (b) no complete or partial response after 2 courses of therapy.	

[a] At least 21 days and until absolute granulocyte count 1000/mm^3.
[b] See text for definition.

grams/100 ml; hepatic function—grade 1 = 50% rise of transaminases above the baseline level, and grade 2 = ≥100% rise above the baseline level; renal function—any rise in BUN and creatinine above normal or a fall in creatinine clearance below normal was considered evidence of renal toxicity; fever—grade 1 = 38°–39°C, and grade 2 = >39°C. Nausea, vomiting, diarrhea, and neuro-

TABLE 2

Summary of Clinical Data and Response to Therapy (5-Azacytidine)

Serial No.	Age (Years)	Sex	Diagnosis (DX)	Site of Primary	Sites of Metastases	Time Since DX (months)	Courses	Dose mg/m²/D	Total > No. of < Courses	Response
1	14	M	OS[a]	Left distal tibia	Lungs	7	1	150	1	Progressive disease, treatment changed
2	15	M	OS[a]	Left proximal fibula	Lungs	8	1 2 3 4	150 200 150 200	4	Progressive disease, treatment changed
3	17	M	OS[a]	Right distal femur	Lungs	4	1 2 3	150 200 200	3	Progressive disease, treatment changed
4	16	M	OS[a]	Left proximal humerus	Lungs, skin, subcutaneous, brain	15	1 2	150 200	2	Progressive disease, patient expired
5	32	M	OS[a]	Left mid humerus	Lungs multiple bones	28	1 2	150 200	2	Progressive disease, patient expired
6	17	M	OS[a]	Left femur	Lungs	10	1	150	1	Progressive disease, patient expired
7	39	F	OS[a]	Pelvis	Lungs, heart, multiple bones	14	1 2	150 150	2	Progressive disease, patient expired
8	16	F	ES[b]	Right proximal tibia	Lungs, multiple bones, bone marrow	13	1 2 3	150 200 200	3	Stable disease between 1st and 2nd course, progression after 2nd course, treatment changed
9	31	M	ES[b]	Rt. fibula	Multiple bones, soft tissues, pleura	20	1	150	1	Progressive disease, patient expired
10	18	M	ES[b]	Ribs	Multiple bones	13	1 2	150 150	2	Progressive disease, patient expired
11	17	F	ES[b]	Left distal humerus	Multiple bones	42	1	150	1	Progressive disease, patient expired
12	19	F	ES[b]	Lumbar vertebra	Multiple bones, bone marrow	7	1 2	150 200	2	Progressive disease, patient expired
13	19	M	ES[b]	Left distal femur	Multiple bones, lungs	8	1 2	150 200	2	Progressive disease, patient expired
14	44	M	ES[b]	Right distal radius	Lungs, brain	54	1	150	1	Progressive disease, patient expired

M = Male.
F = Female.
OS = Osteogenic Sarcoma.
ES = Ewing's Sarcoma.

TABLE 3

Summary of Toxicity

Side Effects and Toxic Effects	Grade[a]	No. of courses[b] in which toxicity noted	No. of pts. with toxicity/ No. of pts. in the study
Nausea	1	17	14/14
and vomiting	2	9	
	3	1	
Fever	1	8	10/14
	2	5	
Hematopoeitic			
Neutropenia	1	6	11/14
	2	6	
Thrombocytopenia	1	2	6/14
	2	3	
	3	2	
Decrease	1	3	4/14
in hemoglobin	2	1	
Hepatic	1	2	3/14
	2	1	
Renal		1	1/14

[a] See text for grading of toxicity.

[b] Total number of courses received by the 14 evaluable patients in the study was 27.

muscular symptoms were graded as mild (grade 1); moderate (grade 2) and severe (grade 3) by the investigators.

Results

Of the 15 patients entered on study, one patient unexpectedly died of his disease while receiving the first course of 5-azacytidine, leaving 14 patients (7 with osteogenic sarcoma and 7 with Ewing's sarcoma) evaluable for toxicity and response.

As shown in Table 2, no patient experienced objective improvement during treatment with 5-azacytidine. All patients showed evidence of progression of disease following one or two courses of therapy.

The toxicities seen in this study are summarized in Table 3. The nadir of neutropenia and thrombocytopenia was 15–21 days, with recovery in 7–14 days. Three patients required platelet transfusion support. Hepatic and renal dysfunction occurred in a minority of patients and was reversible. Neuromuscular symptoms experienced by three patients were difficult to evaluate because of the extensive bone involvement in two patients, and central nervous system involvement in one.

Discussion

5-Azacytidine given as a single agent intravenously, at a dosage of 150–200 mg/m^2/day in 3 divided doses as 5-day courses, had no demonstrable antitumor activity against osteogenic sarcoma or Ewing's sarcoma in patients with advanced metastatic disease. 150 mg/m^2 was chosen as the starting dose of 5-azacytidine in this study, as the maximally tolerated dose on a daily × 5 schedule is 150–200 mg/m^2 in patients with acute leukemia.[6,11] The total daily dose was given as 3 divided doses on a q 8 hour schedule in an attempt to minimize side effects. The infusions were completed in 3 hours.

The major limitation of the drug in most clinical trials has been its toxicity, particularly gastrointestinal. Although 100% of patients in this study experienced nausea and vomiting, it was severe only in one case, and was easily managed with antiemetics and fluids in the remainder of the study patients. Hematologic toxicity in our patients is comparable to that in other studies.[1,3,9,12] None of the deaths in this study was directly attributable to drug toxicity. One patient experienced reversible renal dysfunction similar to that previously reported.[4] Side effects such as skin rash, stomatitis and diarrhea, reported in other studies,[6,10,12] were not observed in our patients.

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Blood (ASH Annual Meeting Abstracts) 2006 108: Abstract 4850

Abstracts Not Selected for Presentation

Development of an Oral Dosage Form of Azacitidine: Overcoming Challenges in Chemistry, Formulation, and Bioavailability.

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Abstract

Azacitidine (Vidaza®), an epigenetic modifier which exerts its therapeutic effect through gene demethylation, is currently approved in a subcutaneous (sc) dosage form for the treatment of myelodysplastic syndromes (MDS). Low-dose, chronic demethylation may lead to improved antiproliferative activity while minimizing side effects. Such chronic demethylation would require a convenient, more frequent dosing schedule. It has, therefore, been an objective of Pharmion Corporation to develop an oral dosage form of azacitidine that could improve the safety and efficacy attributes of the parenterally administered formulation plus have desirable pharmacokinetic characteristics. Azacitidine presents several significant challenges with respect to oral administration including sub-optimal physiochemical characteristics, hydrolytic instability, and active enzymatic degradation - all non-conducive to high passive intestinal tract absorption. Moreover, the drug requires formulated tablet strengths accommodating widely flexible treatment regimens yet must be formulated to avoid the chemical and enzymatic degradation occurring presystemically. Additionally, nonclinical testing of azacitidine bioavailability is hampered by inappropriate animal models representing human gastrointestinal tracts conditions, low tolerability of the drug in several animal species, widely variable pharmacokinetic behavior, and lack of highly sensitive bioanalytical methods for measuring the drug. Data on orally administered azacitidine are sparse but hint at some degree of bioavailability in mice where multiple oral dosing with the drug resulted in lower LD50 values than multiple dosing by the intravenous route (data on file at Pharmion). A published report following long term oral dosing (0.2 mg/kg/day of azacitidine plus 200 mg tetrahydrouridine 3 days per week) in a patient with sickle cell disease resulted in significant increases in total and fetal hemoglobin suggesting absorption of the drug followed by systemic effects (Dover, 1985). A study recently conducted in dogs given azacitidine orally (6 mg/kg) compared to sc and iv (2 mg/kg) showed the drug was absorbed rapidly by the oral route (T_{max}, 15 min) with absolute bioavailability of 67% (compared to 71% following sc dosing). Additional *in vitro* and *ex vivo* characterization of azacitidine stability and permeability have been performed. Data from these studies have been utilized to develop a solid oral dosage form of azacitidine that will move forward into additional animal testing and clinical evaluation. Highly sensitive bioanalytical methods have also been developed for the quantitation of azacitidine in dog and human plasma. A single oral dose escalation study will be conducted in patients to assess the safety, tolerability, and pharmacokinetics of orally administered azacitidine. Data will be evaluated continuously during the dose escalation study. Information generated from these studies will be used to appropriately design a full oral azacitidine clinical development program.

Footnotes

* Corresponding author

Disclosures: Authors Stoltz and Etter are employees of the sponsoring pharmaceutical company.; Authors Obradovic and Jia served as consultants to the sponsoring pharmaceutical company.; Authors Stoltz and Etter have stock options in the sponsoring pharmaceutical company.

American Association for Cancer Research
64th Annual Meeting, April 11-13, Atlantic City, NJ

Abstract # 385

Clinical trial of 5-azacytidine (5-azaCR)

Tan,C.; Burchenal,J.H.; Clarkson,B.; Feinstein,M.; Garcia,E.; Sidhu,J.; Krakoff,I.H.

5-azaCR, an antagonist of cytidine, is active against lines of leukemias L1210 and P815 resistant to Ara-C, 6-MP, and vincristine. Its antileukemic effect in mice is potentiated by adriamycin or thioguanine. Thirty patients (21 adults, 9 children) nrc treated with intravenous 5-azaCR. There were 14 leukemias: 7 ALL, 6 AML, 1 blastic phase CML; 4 lymphomas; 4 melanomas; 6 carcinomas and sarcomas. All had advanced disease and extensive prior chemotherapy, including Ara-C. Twenty-four patients received a total of 40 adequate courses. The dose schedule varied: 45-90 mg/m2/day x 5-11 for solid tumors, and 120-240 mg/m2/day x 5-20, 300 mg/m2 /day x 5-10, and 200 mg/m2 bid x 5 for leukemias. Leukopenia and thrombocytopenia occurred 9-23 days after the start of treatment and recovered within 30 days. Nausea and vomiting were often severe, but less so with divided doses. Abdominal pain and diarrhea also occurred. Fever occurred in 6 children, sepsis in 3, and pneumonia in 2. Bilirubin and SGOT levels were increased in 4. No regression in solid tumors and lymphomas or remission in CML was seen. One of 7 ALL had good partial and 1 of 6 AML had poor partial remissions. Studies are being continued to determine the optimum dose schedule alone and in combination with other agents. (Supported in part by NCI grants CA-05826, CA-08748, and ACS C1-65N)

Absorption, Distribution, and Excretion of 5-Azacytidine (NSC-102816) in Man [1,2,3]

W. M. Troetel,[4] A. J. Weiss,[5] J. E. Stambaugh,[4,5] J. F. Lauclus,[5,6] and R. W. Manthei [4]

SUMMARY

Patients with advanced cancer were given 5-azacytidine labeled at position 4 with radioactive carbon (^{14}C) by either the intravenous (iv) or subcutaneous (sc) route. Absorption of the drug from the sc injection site was rapid and peak plasma levels were attained within one-half hour. Within 2 hours, the plasma level of radioactivity was approximately equal to that noted in the patients treated iv. The plasma half-life after iv injection was 3.5 hours; after sc administration, the plasma half-life was 4.2 hours. Patients receiving the drug sc excreted less drug in the urine than did those receiving the drug iv. No radioactivity was detected in the expired carbon dioxide when the drug was given by either route. Drug uptake into tumor tissue was always greater than uptake into surrounding normal tissue. The highest concentrations of radioactivity in the tissues were achieved when the drug had been given iv. Traces of radioactivity were still detectable in the tissues for as long as 6 days after administration of the drug. The incorporation of radioactivity into tumor RNA but not into DNA was demonstrated. The maximum level of radioactivity detected in the spinal fluid was equivalent to 0.2 μg of 5-azacytidine per milliliter of fluid.

[Cancer Chemother Rep 56:405–411, 1972]

The cytidine analog, 5-azacytidine, was synthesized by Pískala and Šorm (1) and was shown to be a potent inhibitor of lymphoid leukemia in the AK strain of mice (2). 5-Azacytidine also exhibited pronounced cancerostatic effects in vivo against L1210 leukemia and T4 lymphoma in mice (3) and against Ehrlich ascites tumor in mice (4). Li and coworkers, using the L1210 leukemia system, established that the drug inhibited the incorporation of tritiated thymidine or deoxyadenosine into DNA to a greater extent than it inhibited the incorporation of tritiated uridine into RNA (5). The antimetabolite may act to disrupt protein synthesis by incorporating into messenger RNA, thereby interfering with the translocation process of protein synthesis (6,7).

Studies in mice have indicated that 5-azacytidine tends to concentrate in lymphatic tissues after intraperitoneal (ip) administration and is rapidly excreted as unchanged drug and as metabolites in the urine (8,9). In the present

[1] Received Dec 15, 1971; revised Feb 16, 1972.

[2] Supported by grant CA-06071 and contract NIH-70-2136 from Chemotherapy, National Cancer Institute (NCI), National Institutes of Health.

[3] 5-Azacytidine: *s*-triazin-2(1*H*)-one, 4-amino-1-β-D-ribofuranosyl-. Supplied by the Cancer Therapy Evaluation Branch, Chemotherapy, NCI.

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[6] Supported by Public Health Service Research Fellowship No. 1-F03-CA-52030-01, from the Department of Health, Education, and Welfare.

study, patients with advanced cancer were given 5-azacytidine labeled at position 4 with radioactive carbon (5-azacytidine-4-^{14}C) by either the intravenous (iv) or subcutaneous (sc) route. A comparison of the absorption, distribution, and excretion of the radioactive material in the two treatment groups was made. The incorporation of the antimetabolite into tumor nucleic acids was also investigated.

MATERIALS AND METHODS

^{14}C-labeled 5-azacytidine (fig 1) with a specific activity of 49.1 millicuries/millimol (200 microcuries/mg) was synthesized by the Monsanto Research Corporation, Dayton, Ohio. A radiochemical purity of greater than 98% was confirmed by a radioscan of a chromatogram developed in a system of *n*-butanol saturated with water. The labeled material was stored in a desiccator at −10 C until just prior to use.

Unlabeled 5-azacytidine was mixed with approximately 85 microcuries of radioactive drug and was administered to 11 patients at the doses indicated in table 1. The drug was reconstituted with bacteriostatic water for injection and administered by either the iv or sc route through a Millipore Swinnex-25 disposable sterilized filter (0.22μ). Appropriate informed consent was obtained from all patients treated with the labeled drug.

All patients selected for treatment had normal liver and kidney function and no other coexisting disorder, such as an infectious disease process, was present. Blood specimens were collected at the times indicated in table 2 into

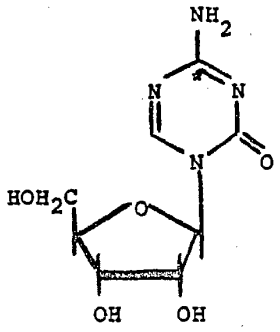

FIGURE 1.—Structure of 5-azacytidine; * indicates position of ^{14}C.

10-ml heparinized glass vacuum tubes. An aliquot of whole blood (0.1 ml) was assayed for radioactivity by combustion in a Packard Tissue Oxidizer (Model 305). The remainder of the blood was centrifuged and the plasma was separated from the packed cell fraction. Plasma radioactivity (0.1-ml sample) was counted in a Packard Liquid Scintillation Spectrometer (Model 3375). A fluor of the following formula was used: 1,4-bis-(2-[5-phenyloxazolyl])-benzene (POPOP), 99 mg; 2,5-diphenyloxazole (PPO), 5.0 g; and Triton X–100 (Packard), 330 ml; diluted with toluene to a total volume of 1000 ml.

Urine was collected at specific intervals for 48–72 hours, the total volume was recorded, and 0.1-ml portions were analyzed for radioactivity in the Triton-X-100-containing fluor described previously. One sample of cerebrospinal fluid (CSF) was obtained from each of four patients at different times and 0.1 ml of each sample was assayed for radioactivity. Samples of ascitic fluid were obtained from only one patient. In all instances, quench corrections were made with an external standard.

Levels of radioactivity in expired carbon dioxide (CO_2) were determined by having patients exhale for 5-minute periods into redistilled ethanolamine. The ethanolamine (4.5 ml) was mixed with 2 volumes of methanol and radioactivity was counted by the addition of 1 volume of fluor composed of PPO, 15 g; *p*-bis-(*o*-methyl-styryl)-benzene (bis-MSB), 1 g; diluted to 1000 ml with toluene. For this assay, counts were corrected by the efficiencies determined with an internal standard of ^{14}C-labeled toluene. Also, CO_2 was collected for 48 hours from male Wistar rats (150–200 g) who had received iv or sc injections of ^{14}C-labeled 5-azacytidine (15 mg/kg, 2 microcuries/animal) in water, and placed in a Nuclear Associates Metabolism Chamber (Model CR 350). The trapping agent for CO_2 was ethanolamine and aliquots (4.5 ml) were assayed for radioactivity as previously described.

For fecal excretion studies, rats received iv or sc injections of labeled 5-azacytidine (15 mg/kg, 2 microcuries/animal) and were housed in individual metabolism cages which permitted the separate collection of urine and feces. The rats were given food and water prior to

TABLE 1.—Patients treated with 5-azacytidine-4-^{14}C

Patient No.	Sex	Age	Weight (kg)	Route of drug administration	Diagnosis
1	F	56	70.5	Iv	Metastatic cystadenocarcinoma of the ovary
2	M	58	75.9	Iv	Metastatic adenocarcinoma of the colon and metastatic melanoma of the retromolar area and buccal mucosa
3	F	57	73.2	Iv	Metastatic cystadenocarcinoma of the ovary
4	F	64	52.3	Iv	Metastatic adenocarcinoma of the breast
5*	M	47	50.5	Iv	Metastatic adenocarcinoma of the rectum
6	M	57	61.4	Iv	Metastatic adenocarcinoma of the colon
7	M	67	54.1	Sc	Metastatic adenocarcinoma of the colon
8	F	45	40.0	Sc	Metastatic adenocarcinoma of the stomach
9	F	63	33.2	Sc	Metastatic squamous cell carcinoma of the lung
10	F	41	89.1	Sc	Metastatic adenocarcinoma of the colon
11	F	38	86.4	Sc	Metastatic synovial sarcoma of the knee

*Received 0.4 mg/kg. All other patients received 1.6 mg/kg.

TABLE 2.—Plasma concentration of 5-azacytidine-4-^{14}C in patients after iv or sc treatment*

Time after administration	Concentration after iv administration in patient No.—							Concentration after sc administration in patient No.—			
	1	2	3	4	5	6	Mean ± SD	7	8	9	Mean ± SD
2 min	11.9	5.74	11.2	—	13.8	4.82	9.49 ± 3.55	—	—	—	—
10 min	2.83	3.44	4.62	4.11	6.64	2.89	4.09 ± 1.31	0.42	—	0.53	0.48
20 min	2.42	—	3.95	3.15	2.60	2.54	2.93 ± 0.60	—	—	—	—
30 min	2.20	2.83	3.54	3.03	2.60	2.13	2.72 ± 0.49	—	2.15	—	—
1 hr	1.91	2.29	3.48	2.65	2.40	1.70	2.41 ± 0.57	1.45	2.07	1.72	1.75 ± 0.25
2 hrs	2.09	1.74	2.61	1.67	1.92	1.41	1.91 ± 0.38	1.37	1.50	1.83	1.57 ± 0.19
4 hrs	1.39	1.24	2.03	—	1.24	1.07	1.39 ± 0.33	1.05	1.22	1.38	1.22 ± 0.14
6 hrs	1.07	0.83	—	—	—	0.98	0.96 ± 0.10	0.87	0.79	—	0.83
12 hrs	0.59	0.30	0.87	0.28	0.30	0.90	0.54 ± 0.27	0.47	0.49	0.55	0.50 ± 0.03
24 hrs	0.29	0.09	0.40	0.08	0.07	0.24	0.20 ± 0.12	0.17	0.31	0.19	0.22 ± 0.06
48 hrs	0.08	—	0	—	—	—	0.04	0.07	0.02	—	0.05

*Values expressed as μg equivalents of 5-azacytidine/ml of plasma.

injection, but after the injection had been given, food but not water was withheld. Feces were collected for 24 and 48 hours after injection. The collected feces were homogenized and weighed, and an aliquot was combusted as described previously and assayed for radioactivity.

Surgically obtained samples of tissue from patients were immediately frozen in glass tubes immersed in a dry ice-acetone bath. Tissues were thawed, rinsed in cold saline, and blotted dry, and total radioactivity was determined by combustion. Uptake of radioactivity into RNA and DNA was determined by the procedure of Wannemacher and co-workers (10). Since the level of radioactivity in the tissue samples was low (*see* table 4), samples were assayed in a liquid scintillation counter preset for 50 minutes or 1000 counts. This setting afforded a standard error in net counting rate of approximately 2%–5%.

RESULTS AND DISCUSSION

Blood

The plasma concentration of radioactivity, expressed as microgram (μg) equivalents of ^{14}C-labeled 5-azacytidine per milliliter of plasma, is shown in table 2. The term "μg equivalent" is defined as the level of radioactivity resulting from drug and metabolites which is equivalent to 1 μg of unchanged 5-azacytidine. The data from patients to whom the drug was given iv (table 2) indicate a triphasic disappearance of radioactivity from plasma. The half-life determined from 30 minutes to 6 hours after iv injection was 3.5 hours. This compared with a half-life of 4.2 hours after sc injection for the same time period. It can be calculated from the data of Raška and co-workers that the half-life of 5-azacytidine and radioactive metabolites in mice is 3.8 hours (8). Thus, there is good correlation between the data reported for mice and the plasma half-life observed in patients. There is no significant difference in plasma levels of radioactivity in patients 2–48 hours after injection regardless of the route of administration. It can be seen also that absorption from the sc site is rapid since a peak level of radioactivity (2.15 μg/ml) was attained in one patient within one-half hour after injection.

Although it was not possible in these studies to determine whether absorption from the sc site in patients was complete, in rats given sc injections of the labeled drug, less than 1% of the administered radioactivity remained at the injection site 2 hours after administration. This was determined by combustion of the skin and subcutaneous tissue from the injection site. Thus, it is likely that the drug is both rapidly and completely absorbed after sc administration.

Ascitic and Cerebrospinal Fluids

Samples of ascitic fluid were obtained from patient No. 1 at half-hour intervals for 6 hours after iv administration of 5-azacytidine. Drug was detectable in the ascitic fluid as early as one-half hour after administration, but did not reach equilibrium with the plasma (1.1 μg/ml) until 6 hours later.

Table 3 indicates that the level of radioactivity in spinal fluid was low when compared with plasma levels. The maximum level of radioactivity in the spinal fluid was noted 24 hours after treatment when the plasma level had declined to 0.4 μg/ml. Since the levels of radioactivity in the CSF were too low to permit identification of the products present, it was not possible to determine whether unchanged drug passed into the spinal fluid. From the limited data available, however, it does appear that significant levels of drug are not attainable in the central nervous system.

Excretion Studies

Figure 2 illustrates the cumulative percent of urinary excretion of radioactivity following iv and sc administration of 5-azacytidine. There was significantly greater excretion of radioactivity over a 48-hour period after iv administration than after sc therapy (85% vs 50%). This was correlated with a higher renal clearance rate in the patients treated iv. Renal clearance of radioactivity was determined in four patients with known normal renal function. Two patients treated iv had renal clearance rates of 76.3 and 127.6 ml/minute and two patients treated sc had renal clearance rates of

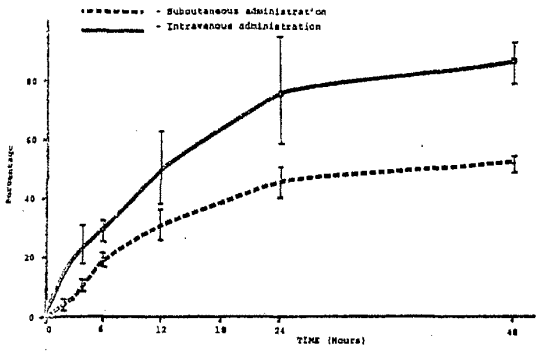

FIGURE 2.—Cumulative percent excretion of urinary radioactivity after the iv and sc administration of 1.6 mg/kg 5-azacytidine to patients. Each point represents the mean ± SD (determined from data for 3–5 patients).

50.4 and 55.2 ml/minute. Since the rate of clearance of radioactivity in all four patients was less than the average glomerular filtration rate of 130 ml/minute, it is apparent that active tubular secretion of labeled substances does not occur (11).

Expired CO_2 from patients was not radioactive regardless of the route of administration of the drug. Forty-eight-hour collections of CO_2 from rats treated iv or sc also were not radioactive. However, any CO_2 produced by the metabolism of 5-azacytidine would probably result from the production of ribosylbiuret (1). Since metabolism to this product would cause the loss of the carbon atom at position 6 of the triazine ring, it would not be detected with the drug labeled at position 4 as in this study.

The rat was used for the determination of the fecal excretion of the drug. Only 3% of the total injected radioactive dose was detected in feces over a 48-hour period after either route of administration. In future studies in patients, the fecal excretion of the drug will be compared with that noted in rats.

Tissue Studies

Levels of radioactivity detected in the tissues of six patients are presented in table 4. The tissue radioactivity is expressed as dpm/g (wet weight) and as the F value as defined in table 4. The latter value permits a comparison of the level of activity in the tissues of patients receiving various doses of radioactive material (12). The data indicate that administration of the drug by the iv route leads to greater tissue uptake and retention of the drug. This is based on the data from samples of carcinoma of the colon from patients Nos. 2, 6, and 10. Specimens of colon from the first two patients treated iv had F values of 0.136 and 0.133 and tissue from the colon of patient No. 10 (treated by the sc route) had an F value of 0.064. These samples of colon were all obtained 44–52 hours after administration of the drug.

In four patients (Nos. 2, 6, 7, and 11), when the carcinoma was removed, adjacent normal tissue was also removed for study. In each case there was a higher uptake of drug in tumor tissue than in the surrounding normal tissue as indicated by the F values in table 4. In patient No. 2, the carcinoma had about 1.3 and 2.5 times the level of radioactivity found in the normal lymph node and appendix respectively. The carcinoma of the colon of patient No. 6 had 1.5 times the radioactivity of normal intestine. Assay of the carcinoma of the lung of patient No. 11 indicated that 2.2 times as much

TABLE 3.—Cerebrospinal fluid (CSF) and plasma levels of radioactivity in patients after iv administration of 5-azacytidine-4-^{14}C

Patient No.	Time after administration (hrs)	CSF*	Plasma*	Plasma-CSF ratio
2	0.75	0.03	2.56	85.3
1	2.0	0.12	1.69	14.0
5	4.5	0.12	1.24	10.3
3	24.0	1.20	0.40	0.33

*Values expressed as μg equivalents of 5-azacytidine-4-^{14}C/ml of spinal fluid and plasma; plasma sample obtained from each patient at same time as spinal fluid sample.

TABLE 4.—Retention of radioactivity in the tissues of patients after iv or sc treatment with 5-azacytidine-4-^{14}C

Patient No.	Tissue	Time after administration (hrs)	Route of administration	Net dpm/g (wet wt)	F value*
2	Carcinoma of the colon	48	Iv	798	0.136
	Normal lymph node			629	0.107
	Normal appendix			319	0.054
	Normal liver			1062	0.181
7	Carcinoma of the colon	30	Sc	416	0.152
	Carcinoma of the colon	144		45	0.016
	Normal intestine			31	0.011
9	Carcinoma of the lung, subcutaneous metastatic thigh lesion	24	Sc	410	0.082
10	Carcinoma of the colon	48	Sc	178	0.064
6	Carcinoma of the colon	48	Iv	371	0.133
	Normal intestine			252	0.090
11	Carcinoma of the lung	48	Sc	170	0.068
	Normal lung			78	0.031
	Normal liver			168	0.067

*F value = $\frac{\text{dpm/g tissue (wet wt)}}{\text{dpm administered per g body wt}}$.

radioactivity had concentrated in the tumor as compared with normal lung. Six days after drug administration, analysis of surgically obtained specimens of the carcinoma of the colon as well as of normal intestine from patient No. 7 indicated that traces of the drug were still retained. The level of radioactivity was low, but the activity in the tumor was approximately 1.5 times greater than that in normal intestine. Thus, regardless of the route of administration, 5-azacytidine tended to concentrate in tumor tissue. When the same types of tumor were compared, however, a higher level of drug uptake after iv administration was evident.

Uptake into the DNA of tumor tissue could not be demonstrated, but incorporation of radioactivity into the RNA was evident as indicated in table 5. The extent of incorporation into RNA ranged from 0.88 to 2.50 dpm/μg RNA. It is of interest to note that the highest specific activity in RNA was determined in patient No. 9 from whom tissues had been obtained 24 hours after injection of 5-azacytidine. The two other patients (Nos. 2 and 6) were treated surgically 48 hours after injection.

Zadražil et al have demonstrated incorporation of 5-azacytidine into the DNA of *E. coli* (13). Li and co-workers reported that 5-azacytidine was incorporated into polynucleotides of the liver, spleen, and ascitic cells of leukemic mice, and into both the RNA (80%–90% total incorporated radioactivity) and the DNA

TABLE 5.—Distribution of radioactivity in tumor after administration of 5-azacytidine-4-^{14}C

Patient No.	Radioactivity in 10% TCA acid-soluble fraction (%)	Radioactivity in RNA (%)	Specific activity of RNA (dpm/μg RNA)	Radioactivity in DNA
2	27.6	77.0	1.18	ND*
6	86.0	18.0	0.88	ND
9	57.0	37.6	2.50	ND

*ND = no radioactivity detectable.

(10%–20%) fractions in L1210 cells in tissue culture (5). Jurovčík et al noted incorporation of 5-azacytidine into the DNA of the thymus and spleen of AKR mice killed 3 hours after they had received ip injections (14). They noted that the level of radioactivity in DNA was substantially lower than the level in RNA and were not certain that the DNA was free of contamination with radioactive RNA. They concluded that proof of incorporation of the drug into DNA in animal systems required further detailed study.

Although we did not detect incorporation of the drug into the DNA of tumor tissue in the patients studied, it is still possible that this may have occurred. Incorporation could occur rapidly and could be followed by disruption of the DNA prior to the 24- and 48-hour sampling periods we used. Future studies of patients from whom tissue can be obtained 2–4 hours after the administration of 5-azacytidine would resolve the problem of whether there is incorporation into DNA followed by a rapid breakdown of the triazine ring. Such samples would also indicate more clearly even minimal incorporation because they would have higher levels of radioactivity.

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Twice Weekly 5-Azacytidine Infusion in Disseminated Metastatic Cancer: A Phase II Study [1,2]

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Writing Committee for the Southeastern Cancer Study Group

SUMMARY

A phase II study of 5-azacytidine given twice weekly as a rapid iv infusion was performed on 116 patients with different metastatic cancers or refractory lymphomas at a dose of 150 mg/m² twice weekly × 6. Ninety-one patients were evaluable. Dose modifications were carried out depending on previous treatment status. Nausea and vomiting was a major side effect; significant granulocytopenia was observed in 35 patients. Responses were observed in only four patients. Our results indicate little effectiveness of this drug. The severe toxicity prevented escalation to potentially more effective dose levels.

[Cancer Treat Rep 61:1675–1677, 1977]

The pyrimidine analog, 5-azacytidine, has been shown to be an effective antineoplastic agent in several tumor systems in experimental animals (1–3). Responses in metastatic cancers have been few (4,5). However, the drug does have activity in acute leukemia (6–8). The studies in L1210 tumor systems indicated that the most effective schedule of administration was by continuous treatment given daily for 4 days, resulting in a 240% increase in lifespan. However, when given every fourth day there was a 207% increase in lifespan indicating considerable activity on that schedule. The Southeastern Cancer Study Group completed a phase I study of twice weekly 5-azacytidine (9); this is a report of a phase II trial in a spectrum of tumors.

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MATERIALS AND METHODS

Patients with disseminated cancer unresponsive to usual modalities of treatment were considered eligible for study provided that their life expectancy was judged to be > 6 weeks and that they had not received more than one prior chemotherapy program, except for patients with lymphoma who were entered in this study only if they were refractory to MOPP or to other forms of equivalent combination chemotherapy. Normal bone marrow and renal and hepatic functions were required. The pretreatment evaluation consisted of a complete clinical history and physical examination with careful measurements of tumor parameters. The following studies were done on all patients: complete blood cell counts; bone marrow; liver profile including transaminases, bilirubin, total proteins, and A/G ratio; serum alkaline phosphatase; and renal function studies including creatinine and BUN. All pertinent studies were repeated during treatment as follows: blood cell counts were performed prior to each dose (twice weekly) and liver function studies were performed once weekly.

Treatment Plan

Patients with metastatic cancers were given 5-azacytidine at a dose of 150 mg/m² twice weekly for 6 weeks which was administered by rapid iv injection dissolved in Ringer's lactate or normal saline. Since we had had very little prior experience with 5-azacytidine in lymphoma patients, we felt that reduced doses were indicated. All patients with lymphoma had had major prior therapy and were given 50 mg/m². No dose escalations were permitted.

Criteria of Response

Measurable tumors were followed carefully to assess any change. An excellent response (complete response) was defined as the disappearance of all evidence of disease. A good response (partial response) indicated > 50% reduction in measurable tumor. A fair response indicated a 25% reduction in tumor size. A questionable response was < 25% reduction.

Toxicity

The hematologic toxicity was judged as follows: *Hemoglobin level*—grade 1 = a fall of 3–4.9 g/100 ml and grade 2 = a fall of ≥ 5 g/100 ml. *Granulocyte count*—grade 1 = a fall to < 1500/mm³; grade 2 = a fall to < 750/mm³; and grade 3 = life-threatening granulocytopenia with infection. *Platelet count*—grade 1 = a fall to < 100,000/mm³; grade 2 = a fall to < 50,000/mm³; and grade 3 = life-threatening.

Other toxicity was graded as follows: *Hepatic function*—grade 1 = a 50% rise of SGOT or alkaline phosphatase above the normal levels and grade 2 = a 100% rise above normal levels. *Renal function*—any rise in BUN or creatinine above normal values was considered evidence of renal toxicity. *Gastrointestinal effects*—nausea and vomiting was graded as mild, moderate, or severe by each investigator.

RESULTS

There were 116 patients entered in this study and 91 were considered evaluable. Of the 25 unevaluable patients, three had an ineligible diagnosis, six had more than a single prior chemotherapy program, seven refused further treatment, five received incorrect doses of 5-azacytidine, one died of intercurrent disease, and three had inadequate data for evaluation. The responses are shown in table 1. Of the 91 evaluable patients, there were only four responses; two were judged as partial response (one large cell carcinoma of the lung and one renal cell carcinoma) and two were judged as fair response (one adenocarcinoma of the lung and one melanoma). Only seven patients with lymphoma were entered at reduced doses and none responded.

Table 2 shows the major toxic side effects. Severe nausea and vomiting occurred in 78% of the patients. Granulocytopenia was noted in 35 patients and was life-threatening in six. Two patients died of infection with low granulocyte counts (< 750 and < 1500/mm³). Significant thrombocytopenia was observed in six patients and in two the count was < 50,000 cells/mm³. Two patients had hemoglobin drops of ≥ 5 g/100 ml and 11 had drops from 3 to 4.9 g/100 ml. There were other minor side effects—one case of hepatic dysfunction and one case of severe alopecia.

Fourteen patients died while receiving treatment, two from toxicity and 12 from progressive disease.

DISCUSSION

This study indicates that 5-azacytidine given on the twice weekly schedule has little effectiveness in disseminated cancer. Severe nausea and vomiting occurred in the majority of patients. Granulocyte toxicity, although significant, was not intolerable in most patients; other hematologic toxicity was mild.

Previous studies concerning the instability of 5-azacytidine in aqueous solution necessitated giving the drug rapidly after reconstitution (10). However, recent studies by Israili et al (11) have indicated that the drug is stable in Ringer's lactate and can be given by continuous infusions. When given in

TABLE 1.—Response to 5-azacytidine

Primary site	No. of patients— Entered	Evaluable	No. of responses
Breast	8	6	0
Renal cell carcinoma	12	10	1 partial response
Other urogenital	3	3	0
Colon	9	7	0
Pancreas	3	2	0
Stomach	4	3	0
Rectum	1	1	0
Lung			
Unspecified	2	2	0
Squamous cell	9	6	0
Adenocarcinoma	13	11	1 FR*
Undifferentiated	5	5	0
Small cell	3	2	0
Large cell	4	2	1 partial response
Melanoma	10	10	1 FR*
Head and neck	14	14	0
Sarcomas, soft tissue	6	3	0
Lymphomas	7	4	0

*FR = 25% reduction in measurable tumor.

TABLE 2.—Major toxic effects (91 evaluable patients)

Toxic effect	Grade*	No. of patients
Nausea and vomiting		79
Granulocytopenia	1	9
	2	20
	3	6
Thrombocytopenia	1	4
	2	2
	3	0
Hemoglobin	1	11
	2	2
	3	0

*See text for definitions.

such a manner, significant antitumor activity has been observed in acute leukemia (8).

Although sufficient numbers of patients were not entered in several of the various signal tumor categories to conclusively exclude activity, the gastrointestinal toxicity seemed to preclude any advantage of giving 5-azacytidine in this fashion. A recent publication would suggest that even continuous infusion of 5-azacytidine has very little activity in solid tumors (12). However, it does remain an active agent in acute leukemia.

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An oral dosage formulation of azacitidine: A pilot pharmacokinetic study.

Sub-category:
Other: leukemia, myelodysplasia and transplantation

Category:
Leukemia, Myelodysplasia and Transplantation

Meeting:
2007 ASCO Annual Meeting

Abstract No:
7084

Citation:
Journal of Clinical Oncology, 2007 ASCO Annual Meeting Proceedings Part I. Vol 25, No. 18S (June 20 Supplement), 2007: 7084

Author(s):
M. R. Ward, M. L. Stoltz, J. B. Etter, L. M. Patton, G. Garcia Manero, S. Sharma

Abstract:

Background: Azacitidine, a cytidine analog, induces DNA demethylation that leads to tumor suppressor gene expression. Cellular effects of azacitidine treatment include differentiation, cell cycle arrest and/or apoptosis. There is a complex, non-linear relationship between plasma exposure and pharmacodynamic effect of azacitidine that can be fully explored with an oral formulation of azacitidine, enabling alternate dosing strategies ranging from intermittent high dose to continuous, low-dose approaches to DNA demethylation. **Methods and Results:** A pharmacokinetic study in dogs given oral azacitidine demonstrated rapid absorption with absolute bioavailability of 67% (compared to 71% following SC dosing). When comparing a single parenteral dose of 75 mg/m^2 (~2 mg/kg) given SC to humans vs. a single oral dose of 16 mg/m^2 (0.8 mg/kg) given to dogs, plasma concentrations of azacitidine were similar despite the 4- to 5-fold difference in dose as calculated by body surface area (BSA). A 14-day toxicology study in dogs evaluated the oral doses of 0.2, 0.4, and 0.8 mg/kg/day. The high dose is the previously identified MTD of 0.55 mg/kg/day based on an oral bioavailability of 67% (approximately equal to 16 mg/m^2/day). Hematologic toxicity, a known and expected effect of azacitidine administered in a repeat-dose regimen was observed at the two highest doses. The oral MTD was determined to be 0.2 mg/kg/day for 14 consecutive days followed by a 21-day recovery period. This provides a cumulative MTD of 2.8 mg/kg for the14 day dosing regimen, which is similar to that seen with IV dosing (2.75 mg/kg over 5 days). Based on the preclinical studies, a multicenter, single-treatment study of oral azacitidine is underway in subjects with MDS, AML, or solid tumors. The trial assesses the safety, tolerability and pharmacokinetics of escalating single doses of orally administered azacitidine. Clinical data from this study will be presented.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION**	see Form PCT/ISA/220 as well as, where applicable, item 5 below.
9516-847-228		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/US2009/002999	14/05/2009	15/05/2008
Applicant		
CELGENE CORPORATION		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**
   a. With regard to the **language,** the international search was carried out on the basis of:
      [X] the international application in the language in which it was filed
      [ ] a translation of the international application into _____, which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b))
   b. [ ] This international search report has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43.6*bis*(a)).
   c. [ ] With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.

2. [ ] **Certain claims were found unsearchable** (See Box No. II)

3. [ ] **Unity of invention is lacking** (see Box No III)

4. With regard to the **title,**
   [X] the text is approved as submitted by the applicant
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   [X] the text is approved as submitted by the applicant
   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority

6. With regard to the **drawings,**
   a. the figure of the **drawings** to be published with the abstract is Figure No. _____
      [ ] as suggested by the applicant
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   b. [X] none of the figures is to be published with the abstract

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
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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

1

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

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2009/002999

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

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

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

Form PCT/ISA/210 (patent family annex) (April 2005)

Electronic Acknowledgement Receipt

EFS ID:	7342673
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
Filer Authorized By:	
Attorney Docket Number:	9516-847-999
Receipt Date:	02-APR-2010
Filing Date:	14-MAY-2009
Time Stamp:	15:52:37
Application Type:	Utility under 35 USC 111(a)

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Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		9516-837-999_IDS.pdf	181303 f46d3b9b88d32a2a28c15be2eecf38ac40f383d4	yes	4

	Multipart Description/PDF files in .zip description				
	Document Description		**Start**	**End**	
	Transmittal Letter		1	1	
	Information Disclosure Statement (IDS) Filed (SB/08)		2	4	
Warnings:					
Information:					
2	Foreign Reference	B01.pdf	3219690 fab93fc3313c65203da596e3e06327366877 3bdb	no	74
Warnings:					
Information:					
3	Foreign Reference	B02.pdf	2806251 825923aa2f5ed46a8a0470a2a8a95da6270 59979	no	85
Warnings:					
Information:					
4		C01_C05.pdf	2803912 5ca53a8f9b6b6873b8c8ce1aaf0eeab68d48 a147	yes	33
	Multipart Description/PDF files in .zip description				
	Document Description		**Start**	**End**	
	NPL Documents		1	7	
	NPL Documents		8	15	
	NPL Documents		16	22	
	NPL Documents		23	28	
	NPL Documents		29	33	
Warnings:					
Information:					
5		C06_C10.pdf	3498108 99c92c832ba7027dd54529761ad3cda0d5c 4e244	yes	47
	Multipart Description/PDF files in .zip description				
	Document Description		**Start**	**End**	
	NPL Documents		1	10	

	NPL Documents		11	17	
	NPL Documents		18	28	
	NPL Documents		29	34	
	NPL Documents		35	47	
Warnings:					
Information:					
6		C11_C15.pdf	3123932 72c23eae90801ca7fc15f32efbf5531e39f52446	yes	36
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	NPL Documents		6	10	
	NPL Documents		11	20	
	NPL Documents		21	26	
	NPL Documents		27	36	
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7		C16_C20.pdf	4470624 ad218cf6e651ff3c0e386d27ebdbb99ab58b1864	yes	55
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	NPL Documents		7	13	
	NPL Documents		14	22	
	NPL Documents		23	31	
	NPL Documents		32	55	
Warnings:					

Information:					
8		C21_C25.pdf	2133493 bc0f7217c4f02919030c7fddce451c7baaf49241	yes	31
	Multipart Description/PDF files in .zip description				
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	NPL Documents		13	18	
	NPL Documents		19	23	
	NPL Documents		24	27	
	NPL Documents		28	31	
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Information:					
9		C26_C30.pdf	2195388 54a881d1a06912c676cf07d8cde8ffb0d3dc7e10	yes	30
	Multipart Description/PDF files in .zip description				
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	NPL Documents		14	23	
	NPL Documents		24	27	
	NPL Documents		28	30	
Warnings:					
Information:					
10		C31_C35.pdf	3214526 86ac4458083438c2b098d4d17e6f250ab601c806	yes	40
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Information:					
11		C36_C40.pdf	959014 950c350e941e008c78484e4daae73922371e9a15	yes	14
	Multipart Description/PDF files in .zip description				
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12	NPL Documents	C41.pdf	117674 d9dd21154843ddf2adccae6e669a0c6850ee98fa	no	4
Warnings:					
Information:					
			Total Files Size (in bytes):	28723915	

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:	Etter *et al.*	Confirmation No.:	5370
Serial No.:	12/466,213	Art Unit:	1623
Filed:	May 14, 2009	Examiner:	To be determined
For:	ORAL FORMULATIONS OF CYTIDINE ANALOGS AND METHODS OF USE THEREOF	Attorney Docket No: (CAM:	9516-847-999 501872-999847)

INFORMATION DISCLOSURE STATEMENT

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, enclosed is a list of sixty (60) references for the Examiner's review and consideration. These references are listed on the enclosed form entitled "List of References Cited by Applicant." These references are listed in chronological or alphabetical order. Copies of these references, except for those of U.S. patent documents A01-17, 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 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 as it is submitted before the mailing of a first Office Action on the merits. However, in the event that any fees are required in connection with this filing, the Director is authorized to charge such fees to Jones Day Deposit Account No. 50-3013 (referencing CAM No. 501872-999847).

Respectfully submitted,

Date April 2, 2010

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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	5370

84802 7590 08/01/2011

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EXAMINER	
CRANE, LAWRENCE E	
ART UNIT	PAPER NUMBER
1623	
MAIL DATE	DELIVERY MODE
08/01/2011	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 _____.

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) *1-65* 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) *1-65* 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 *04/02/2010*.	6) ☐ Other: _____.

The Abstract of the Disclosure is objected to because is does not meet the requirement of the MPEP for US application. Correction is required. See MPEP §608.01(b).

Applicant is reminded of the proper content of an abstract of the disclosure.

In chemical patent abstracts for compounds or compositions, the general nature of the compound or composition should be given as well as its use, *e.g.*, "The compounds are of the class of alkyl benzene sulfonyl ureas, useful as oral anti-diabetics." Exemplifications of a species could be illustrative of members of the class. For processes, the type of reaction, reagents and process conditions should be stated, and generally illustrated by a single example unless variations are necessary. Applicant is respectfully reminded that abstracts are limited to a maximum of 250 words by the MPEP.

Complete revision of the content of the abstract is required on a separate sheet. The present Abstract fails to specify the active ingredient, 5-azacytidine, present in the claims.

This application has been filed with formal drawings acceptable for examination purposes only. However, the brief descriptions thereof are incomplete because while Figures 4, 5,and 6 are described, in the Figures provided there are present figures 4A, 4B, 5A, 5B, 6A and 6B. Examiner respectfully requests a complete description of each separate Figure as an amendment to the disclosure.

The instant disclosure fails to include an up-to-date "Cross-References to Related Applications." See 37 C.F.R. §1.78 and MPEP at §201.11. Applicant is respectfully requested to include the requested information (update to provisional applications status as "expired") as an amendment to the first paragraph of the disclosure.

No claims have been cancelled, no claims have been amended, the disclosure has not been amended, and no new claims have been added as of the date of this Office action. One Information Disclosure Statement (IDS) filed April 2, 2010 has been received with all cited references, annotated, and made of record.

Claims **1-65** 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~~.

The disclosure is objected to because of the following informalities:

Applicant is referred to the comments above concerning the Brief Descriptions of the Figures in re Figures 4, 5, and 6.

Appropriate correction is required.

35 U.S.C. §101 reads as follows:

> "Whoever invents or discovers any new and useful process, machine, manufacture, or composition of matter or any new and useful improvement thereof, may obtain a patent therefore, subject to the conditions and requirements of this title."

Claims **62-65** are rejected under 35 U.S.C. §101 because the claimed recitation of a use, without setting forth any steps involved in the process, results in an improper definition of a process, i.e., results in a claim which is not a proper process claim under 35 U.S.C. §101. See for example *Ex parte Dunki,* 153 USPQ 678 (Bd. App. ,1967) and *Clinical Products, Ltd. v. Brenner,* 255 F. Supp. 131, 149, 149 USPQ 475 (D.D.C. 1966).

In claim **62-65** the terms "Use" and "The use" are derivatives of the verb -- to use --, and in view of the above judicial guidance, examiner respectfully suggests should be replaced with alternative terminology not derived from the noted verb.

Claims **16-22, 45-50 and 53-55** are rejected under 35 U.S.C. §101 because the claim of an *in vivo* result in the present tense implies ownership of the host treated." The above noted limitation by implying ownership of a host required for the observation of a specific pharmacological result is a violation of the 13th and 14th Amendments of the U.S. Constitution, which amendments prohibit the ownership of, or the imposition of involuntary servitude upon without due process of law, any human by another. Applicant is respectfully requested to limit all future method claims to definitions of compounds administered to a host in need thereof for the treatment of a particular disease condition only, and to avoid all process

limitations which must take place following administration of an active ingredient (compound or pharmaceutical composition) to said host.

Examiner respectfully suggests amendment of the claims to replace "achieves" with -- has been show to achieve -- and to replace "subject" with -- test subject -- as one way this rejection may be effectively addressed.

Claims **23-50 and 62-65** are rejected under 35 U.S.C. §112, first paragraph, because the specification, while being enabled for the treatment of a limited number of neoplastic disease conditions (see the non-prospective disclosures in Examples 1-7), does not reasonably provide enablement for the effective treatment of all other "diseases associated with abnormal cell proliferation," including at least cancers of the pancreas (instant Example 11 is entirely prospective), liver and brain. 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 claim **23** is directed to the treatment of " ... all diseases associated with abnormal cell proliferation," a scope of coverage which includes the treatment of many neoplastic and non-neoplastic disease conditions the treatment of which has not bee enabled by the instant disclosed exemplifications.

B. The nature of the invention: The instant invention is directed to the treatment of all " ... diseases associated with abnormal cell proliferation" (see claim **23**) in a host in need thereof by the the administration of an effective dosage of 5-azacytidine, or in the case of claim **36**, an effective dosage of any compound definable as a "cytidine analog."

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 **R**, **S**, and **T**.

D. The level of one or ordinary skill: The factor is not important in the instant analysis.

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 disclosing that 5-azacytidine has neoplastic activity 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.

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.

Claims **1-65** 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 "pharmaceutical composition" implies that the claim will define at least one active ingredient and at least one pharmaceutically acceptable carrier. The latter element is missing from the claim, thereby rendering the claim incomplete. Examiner respectfully suggests incorporation of claim **6** into claim **1** as one possible solution.

In claim **1** at lines 2-3, the term "wherein the composition releases ... to a subject" is a method of treating limitation and therefore has no weight in a pharmaceutical composition claim. Deletion is respectfully requested. See also claims **51 and 62** wherein the same issue reoccurs.

Claims **2-5** lack proper antecedent basis in claim **1** because claim **1** has not defined any of the limitations provided by these claims. Examiner respectfully suggests incorporation of the term of art
-- further comprising -- into each noted claim as one possible solution.

In claim **3** the term "non-enteric coated" renders the instant claim indefinite because the particular coating being applied has not been defined in the claim. See also claim **38**.

In claim **6** the term "an excipient selected from" suggests that only a single one of the subsequently listed materials is the excipient present in the final composition. Inspection of Example 1 (all four herein listed excipients are blended into the final composition) suggests that the noted term may have been intended to read -- at least one excipient selected from --, or perhaps -- one or more excipients selected from --, or the like. Appropriate amendment is respectfully suggested. See also claims **41 and 61** wherein the same issue reoccurs.

In claims **8 and 9** the term "d-alpha" appears to include a technical typographical error. Did applicant intend the term to read
-- D-alpha --? See also claims **34 and 35** wherein the same problem reoccurs.

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 claim **31** wherein the same issue reoccurs.

In claim **23** at line 1, the term "a subject" is incomplete. Examiner respectfully suggests that the noted term should be amended to read
-- a subject in need thereof --.

In claim **23** at line 1-2, the term "a disease associated with abnormal cell proliferation" is indefinite because the disease conditions to be treated have not been specified with adequate particularity. Examiner also notes that there are non-neoplastic diseases (e.g. psoriasis) which

appear to be inappropriately included within the scope of the noted term. See also claims **51 and 62** wherein the same issue reoccurs.

In claim **36** at line 2, the term "cytidine analog" renders the claim improperly dependent because said term fails to further limit the scope of the claim depended from.

The following is a quotation of the appropriate paragraphs of 35 U.S.C. §102 that form the basis for the rejections under this section made in this Office action:

"A person shall be entitled to a patent unless -

(a) the invention was known or used by others in this country, or patented or described in a printed publication in this or a foreign country, before the invention thereof by the applicant for a patent."

(b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States."

(e) the invention was described in

(1) an application for patent described under section 122(b), by another filed in the United States before the invention by the applicant for patent, except that an international application filed under the treaty defined in section 351(a) shall have the effect under this subsection of a national application filed under this subsection of a national application published under section 122(b) only if the international application designating the United States was published under Article 21(2)(a) of such treaty in the English language; or

(2) a patent granted on an application by another filed in the United States before the invention by the applicant for patent, except that a patent shall not be deemed filed in the United States for the purposes of this subsection based on the filing of an international application filed under the treaty defined in section 351(a)."

(f) he did not himself invent the subject matter sought to be patented."

(g)(1) during the course of an interference conducted under section 135 or section 291, another inventor involved therein establishes, to the extent permitted in section 104, that before such person's invention thereof the invention was made by such other inventor and not abandoned suppressed or concealed, or (2) before such person's invention thereof, the invention was made in this country by another inventor who had not abandoned, suppressed or concealed it. In determining priority of invention under this subsection, there shall be considered not only the respective dates of conception and reduction to practice of the invention, but also the reasonable diligence of one who was first to conceive and last to reduce to practice, from a time prior to conception by another.

Claims **1-65** are rejected under 35 U.S.C. §102(**b**) as being anticipated by **Zeldis '740** (PTO-892 ref. **C**: US **7,189,740**).

Applicant is referred to claims ***1 and 7-11*** of the **'740** reference in combination with the **'740** reference disclosures at column 25, lines 8-48 (teachings incluidng pharmaceutical

compositions of 5-azacytidine) and column 7 at lines 16-21 (teaching of the "second active ingredient" to include "conventional therapy for MDS"). Applicant is additionally referred to PTO-892 references **S and T** wherein the administration of 5-azacytidine to a host in need thereof for the treatment of MDS (ref. **S**) and for treatment of neoplastic diseases generally (ref. **T**, entry 890, last line). The second and third references have been cited only to provide examples of MDS and neoplastic therapy in order to provide specific definitions of the generic terms in the **'740** reference. Applicant is also respectfully requested to note that the cited claims in the **'740** reference are directed to the treatment of MDS with pairs of active ingredients, as required by instant claims **12, 31 and 57**.

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 **1-65** 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, a penetration enhancer, and other excipients, and to a method of treating various neoplastic diseases therewith.

Redkar et al. '046 discloses at paragraphs [0003] and [0011], that 5-azacytidine is an active ingredient and that the pharmaceutical compositions thereof are known (paragraphs [0175] to [0182] and are effective in the treatment of neoplastic diseases (Abstract and paragraphs [0031] to [0034]). And at paragraphs [0181] and [0182] this reference teaches the co-administration of 5-azacytidine with a second active ingredient.

Redkar et al. '046 does not expressly disclose pharmaceutical compositions comprising a "permeation enhancer" including the vitamin E derivative TPGS.

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/antineoplastic agents 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.

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. 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 5-azacytidine as disclosed in the two cited U. S. patent publications.

Therefore, the instant claimed method of treating " ... diseases associated with abnormal cell proliferation" including neoplastic diseases by the administration of 5-azacytidine as the active ingredient and TPGS as a permeation enhancer in a 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.

This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. §103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 C.F.R. §1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of 35 U.S.C. §103(c) and potential 35 U.S.C. §§102(f) or (g) prior art under 35 U.S.C. §103(a).

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).

LECrane:lec
07/25/2011

/Lawrence E. Crane/

Primary Examiner, Art Unit 1623

L. E. Crane
Primary Patent Examiner
Technology Center 1600

Notice of References Cited	Application/Control No. 12/466,213	Applicant(s)/Patent Under Reexamination ETTER ET AL.	
	Examiner Lawrence E. Crane	Art Unit 1623	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
*	A	US-2007/0190022 A1	08-2007	Bacopoulos et al.	424/085.1
*	B	US-2006/0074046 A1	04-2006	Redkar et al.	514/049
*	C	US-7,189,740 B2	03-2007	Zeldis, Jerome B.	514/323
	D	US-			
	E	US-			
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	K	US-			
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FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	(R) Dintaman et al., "Inhibition of P-Glycoprotein by D-[alpha]-Tocopherol Polyethylene Glycol 1000 Succinate," Pharmaceutical Research, 16(10), 1550-1556 (1999).
	V	(S) Beers et al. (eds.), Chapter 142, Section 11, in The Merck Manual of Diagnosis and Therapy, 18th Edition, Merck & Co., Inc., Rahway, NJ, January, 2006, only title pages and text pages 1114-1116 supplied.
	W	(T) O'Neil et al. (eds.), "The Merck Index, 14th Edition," Merck & Co., Whitehouse Station, NJ, 2006, only title pages and p. 150 supplied (see Entry 890, "Azacitidine").
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	12466213	ETTER ET AL.
	Examiner	Art Unit
	LAWRENCE E CRANE	1623

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) - no basis for ODP found	7/25/2011	LEC

INTERFERENCE SEARCH			
Class	Subclass	Date	Examiner

	/LAWRENCE E CRANE/ Primary Examiner.Art Unit 1623

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

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	36	✓								

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	12466213	ETTER ET AL.
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✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
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12/466,213 - R1 - Etter et al. - Search Notes(SRNT) - CAPLUS

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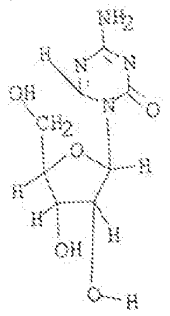

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Absolute stereochemistry.
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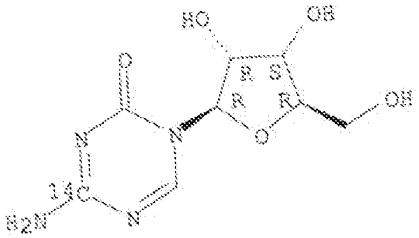


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L7            28 L6 AND MANNITOL

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L7   ANSWER 1 OF 28  CAPLUS  COPYRIGHT 2011 ACS on STN
ED   Entered STN:  28 Apr 2010
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DOCUMENT NUMBER:           152:475600
TITLE:                     Molecular vaccines for infectious disease
INVENTOR(S):               Schoeller, Joergen; Pedersen, Henrik; Brix, Liselotte
PATENT ASSIGNEE(S):        Dako Denmark A/S, Den.
SOURCE:                    PCT Int. Appl., 716pp.
                           CODEN: PIXXD2
DOCUMENT TYPE:             Patent
LANGUAGE:                  English
FAMILY ACC. NUM. COUNT:    3
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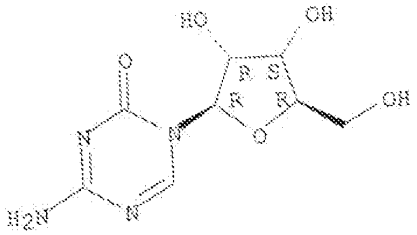
PRIORITY APPLN. INFO.: DK 2008-1384 A 20081002
US 2008-102126P P 20081002
WO 2009-DK50262 TO 20091002

AB The authors disclose the construction of pharmamers (i.e., vaccine components characterized by their multimerization domain and the attached biol. active mols.) and their use in preparation of vaccines that contains the pharmamers alone or in combination with other mols. The individual mols. of the construct can be bound to each other or the multimerization domain(s) by covalent or non-covalent bonds, directly or via linkers. In one example, a pharmamer vaccine targeting immunodeficiency virus is constructed of streptavidin-modified dextran for multimerization of biotinylated MHC class I and class II complexes loaded with peptides from HIV Gag and Env and SIV Nef. [This abstract record is one of three records for this document necessitated by the large number of index entries required to fully index the document and publication system constraints.].

IT 320-67-2
RL: THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(in combination therapy with MHC-based vaccine pharmamers)

RN 320-67-2 CAPLUS
CN 1,3,5-Triazin-2(1H)-one, 4-amino-1-β-D-ribofuranosyl- (CA INDEX NAME)

Absolute stereochemistry.



L7 ANSWER 2 OF 28 CAPLUS COPYRIGHT 2011 ACS on STN
ED Entered STN: 27 Apr 2010
ACCESSION NUMBER: 2010:521732 CAPLUS
DOCUMENT NUMBER: 152:475601
TITLE: Molecular vaccines for infectious disease
INVENTOR(S): Schoeller, Joergen; Pedersen, Henrik; Brix, Liselotte
PATENT ASSIGNEE(S): Dako Denmark A/S, Den.
SOURCE: PCT Int. Appl., 716pp.
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 3
PATENT INFORMATION:

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PRIORITY APPLN. INFO.: DK 2008-1384 A 20081002
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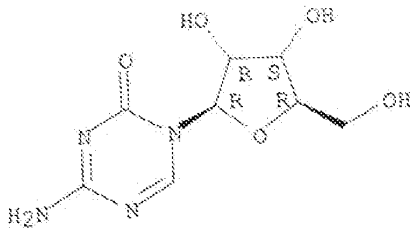
CN 1,3,5-Triazin-2(1H)-one, 4-amino-1-β-D-ribofuranosyl- (CA INDEX NAME)

Absolute stereochemistry.



L7 ANSWER 3 OF 28 CAPLUS COPYRIGHT 2011 ACS on STN
ED Entered STN: 20 Apr 2010
ACCESSION NUMBER: 2010:490042 CAPLUS
DOCUMENT NUMBER: 152:475577
TITLE: MHC multimers in cancer vaccines and immune monitoring
INVENTOR(S): Buus, Soren; Stryhn, Anette; Schoeller, Joergen; Pedersen, Henrik Bria, Liselotte
PATENT ASSIGNEE(S): Dako Denmark A/S, Den.
SOURCE: PCT Int. Appl., 1228pp., MEGA TAN OF 21324085W
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English

FAMILY ACC. NUM. COUNT: 24
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2010037395	A2	20100408	WO 2009-XV50255	20091001

W: 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

RW: 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, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG, BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM

PRIORITY APPLN. INFO.:

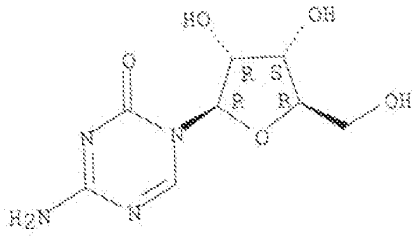
DK 2008-1382	A	20081001
US 2008-101876P	P	20081001
EP 2009-154516	A	20090306

AB The authors disclose the preparation of multimeric MHC-peptide complexes for use in the diagnosis of, treatment of or vaccination against a disease in an individual. In one example, fluorescent dextran conjugates of HLA-A2-peptide complexes are shown to be able to detect antigen-specific T-cells in melanoma patients. [This abstract record is one of 24 records for this document necessitated by the large number of index entries required to fully index the document and publication system constraints.].

IT 320-67-2
RL: THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(in combination therapy with MHC-peptide multimer vaccines)

RN 320-67-2 CAPLUS
CN 1,3,5-Triazin-2(1H)-one, 4-amino-1-β-D-ribofuranosyl- (CA INDEX NAME)

Absolute stereochemistry.



L7 ANSWER 4 OF 28 CAPLUS COPYRIGHT 2011 ACS on STN
ED Entered STN: 20 Apr 2010
ACCESSION NUMBER: 2010:490041 CAPLUS
DOCUMENT NUMBER: 152:499375
TITLE: MHC multimers in cancer vaccines and immune monitoring
INVENTOR(S): Brix, Liselotte; Schoeller, Joergen; Pedersen, Henrik
PATENT ASSIGNEE(S): Dako Denmark A/S, Den.
SOURCE: PCT Int. Appl., 1228pp., MEGA TAN OF 21324085W
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 24
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2010037395	A2	20100408	WO 2009-XL50255	20091001

W: 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
RW: 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, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE,
SN, TD, TG, BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG,
ZM, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM

PRIORITY APPLN. INFO.:

DK 2008-1362	A	20081001
US 2008-101878P	P	20081001
EP 2009-154516	A	20090306

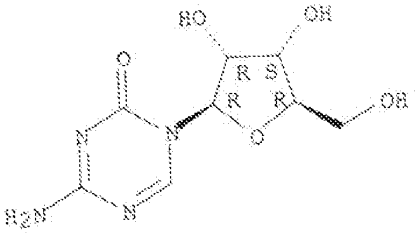
AB The authors disclose the preparation of multimeric MHC-peptide complexes for use in the diagnosis of, treatment of or vaccination against a disease in an individual. In one example, fluorescent dextran conjugates of HLA-A2-peptide complexes are shown to be able to detect antigen-specific T-cells in melanoma patients. [This abstract record is one of 24 records for this document necessitated by the large number of index entries required to fully index the document and publication system constraints.].

IT 320-67-2
RL: THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(in combination therapy with MHC-peptide multimer vaccines)

RN 320-67-2 CAPLUS

CN 1,3,5-Triazin-2(1H)-one, 4-amino-1-β-D-ribofuranosyl- (CA INDEX NAME)

Absolute stereochemistry.



L7 ANSWER 5 OF 20 CAPLUS COPYRIGHT 2011 ACS on STN

ED Entered STN: 20 Apr 2010

ACCESSION NUMBER: 2010:490040 CAPLUS

DOCUMENT NUMBER: 152:475576

TITLE: MHC multimers in cancer vaccines and immune monitoring

INVENTOR(S): Buus, Liselotte; Schoeller, Joergen; Pedersen, Henrik

PATENT ASSIGNEE(S): Dako Denmark A/S, Den.

SOURCE: PCT Int. Appl., 1228pp., MEGA TAN OF 21324085W
CODEN: PIXXD2

DOCUMENT TYPE: Patent

LANGUAGE: English

FAMILY ACC. NUM. COUNT: 24

PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2010037395	A2	20100408	WO 2009-XO50255	20091001

W: 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

RW: 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, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG, BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM

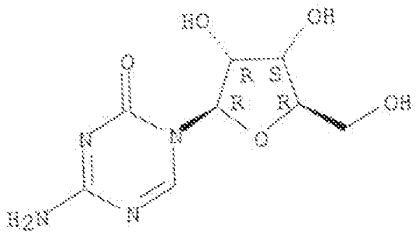
PRIORITY APPLN. INFO.: DK 2008-1382 A 20081001
US 2008-101878P P 20081001
EP 2009-154516 A 20090306

AB The authors disclose the preparation of multimeric MHC-peptide complexes for use in the diagnosis of, treatment of or vaccination against a disease in an individual. In one example, fluorescent dextran conjugates of HLA-A2-peptide complexes are shown to be able to detect antigen-specific T-cells in melanoma patients. [This abstract record is one of 24 records for this document necessitated by the large number of index entries required to fully index the document and publication system constraints.].

IT 320-67-2
RL: THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(in combination therapy with MHC-peptide multimer vaccines)

RN 320-67-2 CAPLUS
CN 1,3,5-Triazin-2(1H)-one, 4-amino-1-β-D-ribofuranosyl- (CA INDEX NAME)

Absolute stereochemistry.



L7 ANSWER 6 OF 28 CAPLUS COPYRIGHT 2011 ACS on STN
ED Entered STN: 20 Apr 2010
ACCESSION NUMBER: 2010:490039 CAPLUS
DOCUMENT NUMBER: 152:452055
TITLE: MHC multimers in cancer vaccines and immune monitoring
INVENTOR(S): Brix, Liselotte; Schoeller, Joergen; Pedersen, Henrik
PATENT ASSIGNEE(S): Dako Denmark A/S, Den.
SOURCE: PCT Int. Appl., 1228pp., MEGA TAN OF 213240858W
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 24
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2010037395	A2	20100408	WO 2009-XC50255	20091001
W: 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				
RW: 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, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG, BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM				
PRIORITY APPLN. INFO.:			DK 2008-1382	A 20081001
			US 2008-101878P	P 20081001
			EP 2009-154516	A 20090306

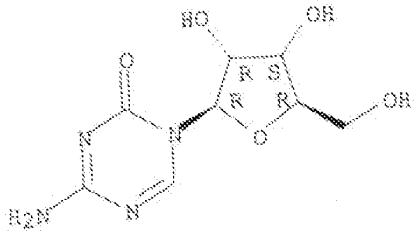
AB The authors disclose the preparation of multimeric MHC-peptide complexes for use in the diagnosis of, treatment of or vaccination against a disease in an individual. In one example, fluorescent dextran conjugates of HLA-A2-peptide complexes are shown to be able to detect antigen-specific T-cells in melanoma patients. [This abstract record is one of 24 records for this document necessitated by the large number of index entries required

to fully index the document and publication system constrain' .].

IT 320-67-2
RL: THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(in combination therapy with MHC-peptide multimer vaccines)

RN 320-67-2 CAPLUS
CN 1,3,5-Triazin-2(1H)-one, 4-amino-1-β-D-ribofuranosyl- (CA INDEX NAME)

Absolute stereochemistry.



L7 ANSWER 7 OF 28 CAPLUS COPYRIGHT 2011 ACS on STN
ED Entered STN: 20 Apr 2010
ACCESSION NUMBER: 2010:490038 CAPLUS
DOCUMENT NUMBER: 152:499374
TITLE: MHC multimers in cancer vaccines and immune monitoring
INVENTOR(S): Brix, Liselotte; Schoeller, Joergen; Pedersen, Henrik
PATENT ASSIGNEE(S): Dako Denmark A/S, Den.
SOURCE: PCT Int. Appl., 1228pp., MEGA TAN OF 213240B5W
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 24
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2010037395	A2	20100408	WO 2009-XS50255	20091001

W: 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

RW: 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, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG, BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM

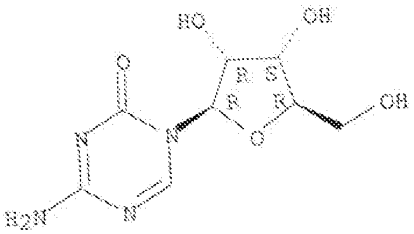
PRIORITY APPLN. INFO.:	DK 2008-1382	A 20081001
	US 2008-101878P	P 20081001
	EP 2009-154516	A 20090306

AB The authors disclose the preparation of multimeric MHC-peptide complexes for use in the diagnosis of, treatment of or vaccination against a disease in an individual. In one example, fluorescent dextran conjugates of HLA-A2-peptide complexes are shown to be able to detect antigen-specific T-cells in melanoma patients. [This abstract record is one of 24 records for this document necessitated by the large number of index entries required to fully index the document and publication system constraints.].

IT 320-67-2
RL: THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(in combination therapy with MHC-peptide multimer vaccines)

RN 320-67-2 CAPLUS
CN 1,3,5-Triazin-2(1H)-one, 4-amino-1-β-D-ribofuranosyl- (CA INDEX NAME)

Absolute stereochemistry.



L7 ANSWER 8 OF 20 CAPLUS COPYRIGHT 2011 ACS on STN
ED Entered STN: 20 Apr 2010
ACCESSION NUMBER: 2010:490037 CAPLUS
DOCUMENT NUMBER: 152:475575
TITLE: MHC multimers in cancer vaccines and immune monitoring
INVENTOR(S): Brix, Liselotte; Schoeller, Joergen; Pedersen, Henrik
PATENT ASSIGNEE(S): Dako Denmark A/S, Den.
SOURCE: PCT Int. Appl., 1228pp., MEGA TAN OF 21324085W
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 24
PATENT INFORMATION:

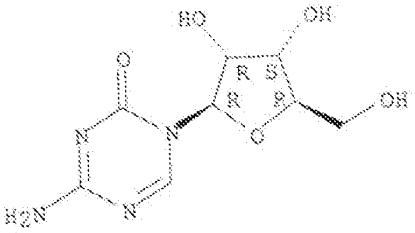
PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2010037395	A2	20100408	WO 2009-XF50255	20091001
W: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, 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			
RW: 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, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG, BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM			
PRIORITY APPLN. INFO.:			DK 2008-1382	A 20081001
			US 2008-101878P	P 20081001
			EP 2009-154516	A 20090306

AB The authors disclose the preparation of multimeric MHC-peptide complexes for use in the diagnosis of, treatment of or vaccination against a disease in an individual. In one example, fluorescent dextran conjugates of HLA-A2-peptide complexes are shown to be able to detect antigen-specific T-cells in melanoma patients. [This abstract record is one of 24 records for this document necessitated by the large number of index entries required to fully index the document and publication system constraints.].

IT 320-67-2
RL: THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(in combination therapy with MHC-peptide multimer vaccines)

RN 320-67-2 CAPLUS
CN 1,3,5-Triazin-2(1H)-one, 4-amino-1-β-D-ribofuranosyl- (CA INDEX NAME)

Absolute stereochemistry.



L7 ANSWER 9 OF 28 CAPLUS COPYRIGHT 2011 ACS on STN
ED Entered STN: 20 Apr 2010
ACCESSION NUMBER: 2010:490036 CAPLUS
DOCUMENT NUMBER: 152:452054
TITLE: MHC multimers in cancer vaccines and immune monitoring
INVENTOR(S): Brix, Liselotte; Schoeller, Joergen; Pedersen, Henrik
PATENT ASSIGNEE(S): Dako Denmark A/S, Den.
SOURCE: PCT Int. Appl., 1228pp., MEGA TAN OF 21324085W
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 24
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2010037395	A2	20100408	WO 2009-KU50255	20091001

W: 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

RW: 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, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG, BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM

PRIORITY APPLN. INFO.:

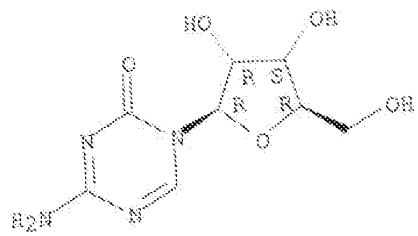
DK 2008-1382	A	20081001
US 2008-101878P	P	20081001
EP 2009-154516	A	20090306

AB The authors disclose the preparation of multimeric MHC-peptide complexes for use in the diagnosis of, treatment of or vaccination against a disease in an individual. In one example, fluorescent dextran conjugates of HLA-A2-peptide complexes are shown to be able to detect antigen-specific T-cells in melanoma patients. {This abstract record is one of 24 records for this document necessitated by the large number of index entries required to fully index the document and publication system constraints.}.

IT 320-67-2
RL: THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(in combination therapy with MHC-peptide multimer vaccines)

RN 320-67-2 CAPLUS
CN 1,3,5-Triazin-2(1H)-one, 4-amino-1-β-D-ribofuranosyl- (CA INDEX NAME)

Absolute stereochemistry.



L7 ANSWER 10 OF 28 CAPLUS COPYRIGHT 2011 ACS on STN
ED Entered STN: 20 Apr 2010
ACCESSION NUMBER: 2010:490035 CAPLUS
DOCUMENT NUMBER: 152:452053
TITLE: MHC multimers in cancer vaccines and immune monitoring
INVENTOR(S): Brix, Liselotte; Schoeller, Joergen; Pedersen, Henrik
PATENT ASSIGNEE(S): Dako Denmark A/S, Den.
SOURCE: PCT Int. Appl., 1228pp., MEGA TAN OF 21324085W
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 24
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2010037395	A2	20100408	WO 2009-XH50255	20091001

W: 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, SE, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW
RW: 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, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG, BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM

PRIORITY APPLN. INFO.:

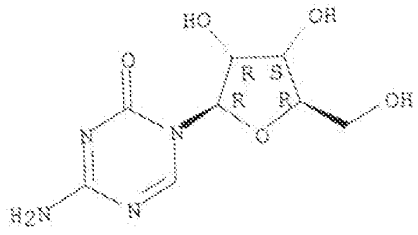
DK 2008-1382	A	20081001
US 2008-101878P	P	20081001
EP 2009-154516	A	20090306

AB The authors disclose the preparation of multimeric MHC-peptide complexes for use in the diagnosis of, treatment of or vaccination against a disease in an individual. In one example, fluorescent dextran conjugates of HLA-A2-peptide complexes are shown to be able to detect antigen-specific T-cells in melanoma patients. [This abstract record is one of 24 records for this document necessitated by the large number of index entries required to fully index the document and publication system constraints.].

IT 320-67-2
RL: THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(in combination therapy with MHC-peptide multimer vaccines)

RN 320-67-2 CAPLUS
CN 1,3,5-Triazin-2(1H)-one, 4-amino-1-β-D-ribofuranosyl- (CA INDEX NAME)

Absolute stereochemistry.



L7 ANSWER 11 OF 28 CAPLUS COPYRIGHT 2011 ACS on STN
ED Entered STN: 20 Apr 2010
ACCESSION NUMBER: 2010:490034 CAPLUS
DOCUMENT NUMBER: 152:499373
TITLE: MHC multimers in cancer vaccines and immune monitoring
INVENTOR(S): Brix, Liselotte; Schoeller, Joergen; Pedersen, Henrik
PATENT ASSIGNEE(S): Dako Denmark A/S, Den.
SOURCE: PCT Int. Appl., 1228pp., MEGA TAN OF 21324085W

CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 24
PATENT INFORMATION:

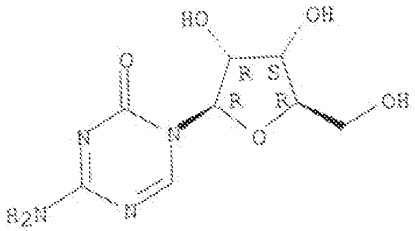
PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2010037395	A2	20100408	WO 2009-XM50255	20091001
W: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, 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				
RW: 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, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG, BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM				
PRIORITY APPLN. INFO.:			DK 2008-1382	A 20081001
			US 2008-101878P	P 20081001
			EP 2009-154516	A 20090306

AB The authors disclose the preparation of multimeric MHC-peptide complexes for use in the diagnosis of, treatment of or vaccination against a disease in an individual. In one example, fluorescent dextran conjugates of HLA-A2-peptide complexes are shown to be able to detect antigen-specific T-cells in melanoma patients. [This abstract record is one of 24 records for this document necessitated by the large number of index entries required to fully index the document and publication system constraints.].

IT 320-67-2
RL: THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(in combination therapy with MHC-peptide multimer vaccines)

RN 320-67-2 CAPLUS
CN 1,3,5-Triazin-2(1H)-one, 4-amino-1-β-D-ribofuranosyl- (CA INDEX NAME)

Absolute stereochemistry.



L7 ANSWER 12 OF 28 CAPLUS COPYRIGHT 2011 ACS on STN
ED Entered STN: 20 Apr 2010
ACCESSION NUMBER: 2010:490033 CAPLUS
DOCUMENT NUMBER: 152:427625
TITLE: MHC multimers in cancer vaccines and immune monitoring
INVENTOR(S): Brix, Liselotte; Schoeller, Joergen; Pedersen, Henrik
PATENT ASSIGNEE(S): Dako Denmark A/S, Den.
SOURCE: PCT Int. Appl., 1228 pp., MEGA TAN OF 21324085W
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 24
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2010037395	A2	20100408	WO 2009-XM50255	20091001

W: 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
RW: 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, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE,
SN, TD, TG, BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG,
ZM, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM

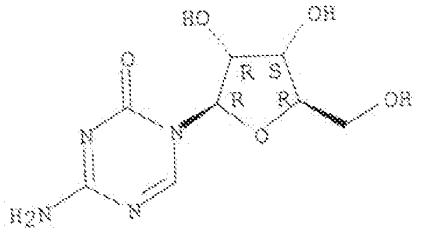
PRIORITY APPLN. INFO.: DK 2008-1382 A 20081001
US 2008-101878P P 20081001
EP 2009-154516 A 20090306

AB The authors disclose the preparation of multimeric MHC-peptide complexes for use in the diagnosis of, treatment of or vaccination against a disease in an individual. In one example, fluorescent dextran conjugates of HLA-A2-peptide complexes are shown to be able to detect antigen-specific T-cells in melanoma patients. [This abstract record is one of 24 records for this document necessitated by the large number of index entries required to fully index the document and publication system constraints.].

IT 320-67-2
RL: THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(in combination therapy with MHC-peptide multimer vaccines)

RN 320-67-2 CAPLUS
CN 1,3,5-Triazin-2(1H)-one, 4-amino-1-β-D-ribofuranosyl- (CA INDEX NAME)

Absolute stereochemistry.



L7 ANSWER 13 OF 28 CAPLUS COPYRIGHT 2011 ACS on STN
ED Entered STN: 20 Apr 2010
ACCESSION NUMBER: 2010:490032 CAPLUS
DOCUMENT NUMBER: 152:475574
TITLE: MHC multimers in cancer vaccines and immune monitoring
INVENTOR(S): Brix, Liselotte; Schoeller, Joergen; Pedersen, Henrik
PATENT ASSIGNEE(S): Dako Denmark A/S, Den.
SOURCE: PCT Int. Appl., 1228pp., MEGA TAN OF 21324085W
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 24
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2010037395	A2	20100408	WO 2009-XW50255	20091001

W: 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
RW: 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, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE,
SN, TD, TG, BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG,
ZM, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM

PRIORITY APPLN. INFO.:

DK 2008-1382	A	20081001
US 2008-101878P	P	20081001
EP 2009-154516	A	20090306

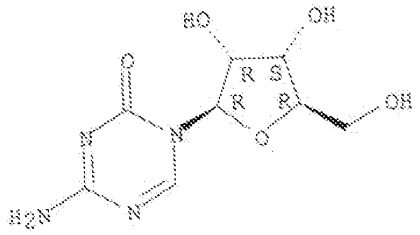
AB The authors disclose the preparation of multimeric MHC-peptide complexes for use in the diagnosis of, treatment of or vaccination against a disease in an individual. In one example, fluorescent dextran conjugates of HLA-A2-peptide complexes are shown to be able to detect antigen-specific T-cells in melanoma patients. [This abstract record is one of 24 records for this document necessitated by the large number of index entries required to fully index the document and publication system constraints.].

IT 320-67-2
RL: THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(in combination therapy with MHC-peptide multimer vaccines)

RN 320-67-2 CAPLUS

CN 1,3,5-Triazin-2(1H)-one, 4-amino-1-β-D-ribofuranosyl- (CA INDEX NAME)

Absolute stereochemistry.



L7 ANSWER 14 OF 28 CAPLUS COPYRIGHT 2011 ACS on STN

ED Entered STN: 20 Apr 2010

ACCESSION NUMBER: 2010:490031 CAPLUS

DOCUMENT NUMBER: 152:452052

TITLE: MHC multimers in cancer vaccines and immune monitoring

INVENTOR(S): Brix, Liselotte; Schoeller, Joergen; Pedersen, Henrik

PATENT ASSIGNEE(S): Dako Denmark A/S, Den.

SOURCE: PCT Int. Appl., 1228pp., MEGA TAN OF 21324085W
CODEN: PIXXD2

DOCUMENT TYPE: Patent

LANGUAGE: English

FAMILY ACC. NUM. COUNT: 24

PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2010037395	A2	20100408	WO 2009-XIS0255	20091001

W: 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

RW: 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, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG, BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM

PRIORITY APPLN. INFO.:

DK 2008-1382	A	20081001
US 2008-101878P	P	20081001
EP 2009-154516	A	20090306

AB The authors disclose the preparation of multimeric MHC-peptide complexes for use in the diagnosis of, treatment of or vaccination against a disease in an individual. In one example, fluorescent dextran conjugates of

HLA-A2-peptide complexes ar- shown to be able to detect anti-n-specific
T-cells in melanoma patieni [This abstract record is one 24 records
for this document necessitated by the large number of index entries required
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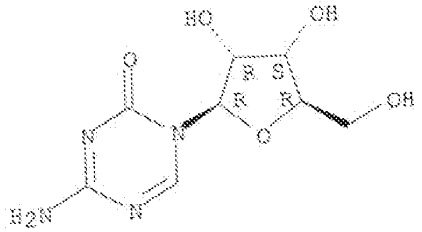
IT 320-67-2
RL: THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(in combination therapy with MHC-peptide multimer vaccines)

RN 320-67-2 CAPLUS
CN 1,3,5-Triazin-2(1H)-one, 4-amino-1-β-D-ribofuranosyl- (CA INDEX NAME)

Absolute stereochemistry.



L7 ANSWER 15 OF 20 CAPLUS COPYRIGHT 2011 ACS on STN
ED Entered STN: 20 Apr 2010
ACCESSION NUMBER: 2010:490030 CAPLUS
DOCUMENT NUMBER: 152:475573
TITLE: MHC multimers in cancer vaccines and immune monitoring
INVENTOR(S): Brix, Liselotte; Schoeller, Joergen; Pedersen, Henrik
PATENT ASSIGNEE(S): Dako Denmark A/S, Den.
SOURCE: PCT Int. Appl., 1228pp., MEGA TAN OF 21324085W
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 24
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2010037395	A2	20100408	WO 2009-XA50255	20091001

W: 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

RW: 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, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG, BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM

PRIORITY APPLN. INFO.:

DK 2008-1382	A	20081001
US 2008-101878P	P	20081001
EP 2009-154516	A	20090306

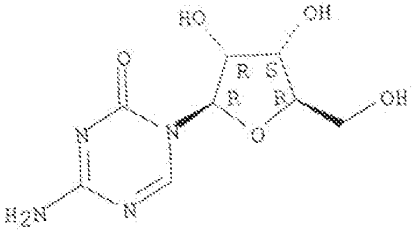
AB The authors disclose the preparation of multimeric MHC-peptide complexes for use in the diagnosis of, treatment of or vaccination against a disease in an individual. In one example, fluorescent dextran conjugates of HLA-A2-peptide complexes are shown to be able to detect antigen-specific T-cells in melanoma patients. [This abstract record is one of 24 records for this document necessitated by the large number of index entries required to fully index the document and publication system constraints.].

IT 320-67-2
RL: THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(in combination therapy with MHC-peptide multimer vaccines)

RN 320-67-2 CAPLUS

CN 1,3,5-Triazin-2(1H)-one, 4-~~ino-1-β-D-ribofuranosyl- (CA I~~EX NAME)

Absolute stereochemistry.



L7 ANSWER 16 OF 28 CAPLUS COPYRIGHT 2011 ACS on STN
ED Entered STN: 20 Apr 2010
ACCESSION NUMBER: 2010:490029 CAPLUS
DOCUMENT NUMBER: 152:439372
TITLE: MHC multimers in cancer vaccines and immune monitoring
INVENTOR(S): Bris, Liselotte; Schoellier, Joergen; Pedersen, Henrik
PATENT ASSIGNEE(S): Dako Denmark A/S, Den.
SOURCE: PCT Int. Appl., 1228pp., MEGA TAN OF 21324085W
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 24
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2010037395	A2	20100408	WO 2009-XR50255	20091001

W: 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

RW: 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, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG, BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM

PRIORITY APPLN. INFO.:

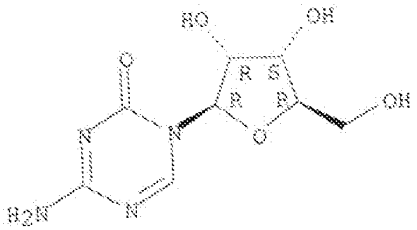
DK 2008-1382	A	20081001
US 2008-101878P	P	20081001
EP 2009-154516	A	20090306

AB The authors disclose the preparation of multimeric MHC-peptide complexes for use in the diagnosis of, treatment of or vaccination against a disease in an individual. In one example, fluorescent dextran conjugates of HLA-A2-peptide complexes are shown to be able to detect antigen-specific T-cells in melanoma patients. [This abstract record is one of 24 records for this document necessitated by the large number of index entries required to fully index the document and publication system constraints.].

IT 320-67-2
RL: THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(in combination therapy with MHC-peptide multimer vaccines)

RN 320-67-2 CAPLUS
CN 1,3,5-Triazin-2(1H)-one, 4-amino-1-β-D-ribofuranosyl- (CA INDEX NAME)

Absolute stereochemistry.



L7 ANSWER 17 OF 28 CAPLUS COPYRIGHT 2011 ACS on STN
ED Entered STN: 20 Apr 2010
ACCESSION NUMBER: 2010:490028 CAPLUS
DOCUMENT NUMBER: 152:499371
TITLE: MHC multimers in cancer vaccines and immune monitoring
INVENTOR(S): Brix, Liselotte; Schoeller, Joergen; Pedersen, Henrik
PATENT ASSIGNEE(S): Dako Denmark A/S, Den.
SOURCE: PCT Int. Appl., 1228pp., MEGA TAN OF 21324085W
CODEN: PIXXD2

DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 24
PATENT INFORMATION:

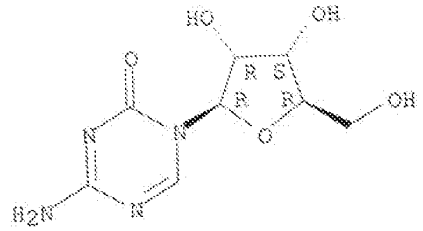
PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2010037395	A2	20100408	WO 2009-XN50255	20091001
W: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, 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				
RW: 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, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG, BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM				
PRIORITY APPLN. INFO.:			DK 2008-1392	A 20081001
			US 2008-101878P	P 20081001
			EP 2009-154516	A 20090306

AB The authors disclose the preparation of multimeric MHC-peptide complexes for use in the diagnosis of, treatment of or vaccination against a disease in an individual. In one example, fluorescent dextran conjugates of HLA-A2-peptide complexes are shown to be able to detect antigen-specific T-cells in melanoma patients. [This abstract record is one of 24 records for this document necessitated by the large number of index entries required to fully index the document and publication system constraints.].

IT 320-67-2
RL: THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(in combination therapy with MHC-peptide multimer vaccines)

RN 320-67-2 CAPLUS
CN 1,3,5-Triazin-2(1H)-one, 4-amino-1-β-D-ribofuranosyl- (CA INDEX NAME)

Absolute stereochemistry.



L7 ANSWER 18 OF 28 CAPLUS © RIGHT 2011 ACS on STN
ED Entered STN: 20 Apr 2010
ACCESSION NUMBER: 2010:490027 CAPLUS
DOCUMENT NUMBER: 152:499370
TITLE: MHC multimers in cancer vaccines and immune monitoring
INVENTOR(S): Brix, Liselotte; Schoeller, Joergen; Pedersen, Henrik
PATENT ASSIGNEE(S): Dako Denmark A/S, Den.
SOURCE: PCT Int. Appl., 1228pp., MEGA TAN OF 21324085W
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 24
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2010037395	A2	20100408	WO 2009-EP50255	20091001

W: 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

RW: 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, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG, BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM

PRIORITY APPLN. INFO.:

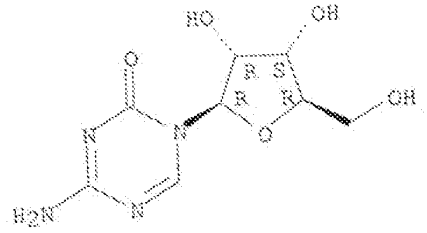
DK 2008-1382	A	20081001
US 2008-101878P	P	20081001
EP 2009-154516	A	20090306

AB The authors disclose the preparation of multimeric MHC-peptide complexes for use in the diagnosis of, treatment of or vaccination against a disease in an individual. In one example, fluorescent dextran conjugates of HLA-A2-peptide complexes are shown to be able to detect antigen-specific T-cells in melanoma patients. [This abstract record is one of 24 records for this document necessitated by the large number of index entries required to fully index the document and publication system constraints.].

IT 320-67-2
RL: THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(in combination therapy with MHC-peptide multimer vaccines)

RN 320-67-2 CAPLUS
CN 1,3,5-Triazin-2(1H)-one, 4-amino-1-β-D-ribofuranosyl- (CA INDEX NAME)

Absolute stereochemistry.



L7 ANSWER 19 OF 28 CAPLUS COPYRIGHT 2011 ACS on STN
ED Entered STN: 20 Apr 2010
ACCESSION NUMBER: 2010:490026 CAPLUS
DOCUMENT NUMBER: 152:499369
TITLE: MHC multimers in cancer vaccines and immune monitoring
INVENTOR(S): Brix, Liselotte; Schoeller, Joergen; Pedersen, Henrik
PATENT ASSIGNEE(S): Dako Denmark A/S, Den.
SOURCE: PCT Int. Appl., 1228pp., MEGA TAN OF 21324085W

CODEN: ""XXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 24
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2010037395	A2	20100408	WO 2009-XQ50255	20091001

W: 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

RW: 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, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG, BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM

PRIORITY APPLN. INFO.:

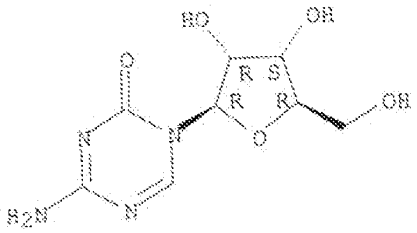
DK 2008-1362	A	20081001
US 2008-101878P	P	20081001
EP 2009-154516	A	20090306

AB The authors disclose the preparation of multimeric MHC-peptide complexes for use in the diagnosis of, treatment of or vaccination against a disease in an individual. In one example, fluorescent dextran conjugates of HLA-A2-peptide complexes are shown to be able to detect antigen-specific T-cells in melanoma patients. [This abstract record is one of 24 records for this document necessitated by the large number of index entries required to fully index the document and publication system constraints.].

IT 320-67-2
RL: THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(in combination therapy with MHC-peptide multimer vaccines)

RN 320-67-2 CAPLUS
CN 1,3,5-Triazin-2(1H)-one, 4-amino-1-β-D-ribofuranosyl- (CA INDEX NAME)

Absolute stereochemistry.



L7 ANSWER 20 OF 28 CAPLUS COPYRIGHT 2011 ACS on STN
ED Entered STN: 20 Apr 2010
ACCESSION NUMBER: 2010:490025 CAPLUS
DOCUMENT NUMBER: 152:499368
TITLE: MHC multimers in cancer vaccines and immune monitoring
INVENTOR(S): Brix, Liselotte; Schoeller, Joergen; Pedersen, Henrik
PATENT ASSIGNEE(S): Dako Denmark A/S, Den.
SOURCE: PCT Int. Appl., 1228pp., MEGA TAN OF 21324085W
CODEN: PIXXD2

DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 24
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2010037395	A2	20100408	WO 2009-XQ50255	20091001

W: 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
RW: 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, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE,
SN, TD, TG, BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG,
ZM, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM

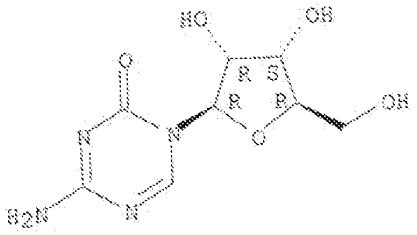
PRIORITY APPLN. INFO.: DK 2008-1382 A 20081001
US 2008-101878P P 20081001
EP 2009-154516 A 20090306

AB The authors disclose the preparation of multimeric MHC-peptide complexes for use in the diagnosis of, treatment of or vaccination against a disease in an individual. In one example, fluorescent dextran conjugates of HLA-A2-peptide complexes are shown to be able to detect antigen-specific T-cells in melanoma patients. [This abstract record is one of 24 records for this document necessitated by the large number of index entries required to fully index the document and publication system constraints.].

IT 320-67-2
RL: THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(in combination therapy with MHC-peptide multimer vaccines)

RN 320-67-2 CAPLUS
CN 1,3,5-Triazin-2(1H)-one, 4-amino-1-β-D-ribofuranosyl- (CA INDEX NAME)

Absolute stereochemistry.



L7 ANSWER 21 OF 28 CAPLUS COPYRIGHT 2011 ACS on STN
ED Entered STN: 20 Apr 2010
ACCESSION NUMBER: 2010:490024 CAPLUS
DOCUMENT NUMBER: 152:499367
TITLE: MHC multimers in cancer vaccines and immune monitoring
INVENTOR(S): Brix, Liselotte; Schoeller, Joergen; Pedersen, Henrik
PATENT ASSIGNEE(S): Dako Denmark A/S, Den.
SOURCE: PCT Int. Appl., 1226pp., MEGA TAN OF 21324085W
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 24
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2010037395	A2	20100408	WO 2009-XB50255	20091001

W: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ,
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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
RW: 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, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE,
SN, TD, TG, BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG,
ZM, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM

PRIORITY APPLN. INFO.:

DK 2008-1382	A	20081001
US 2008-101878P	P	20081001
EP 2009-154516	A	20090306

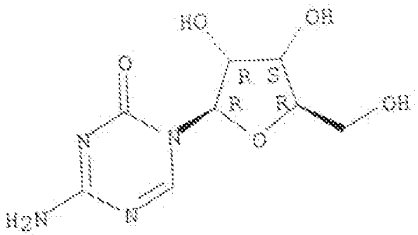
AB The authors disclose the preparation of multimeric MHC-peptide complexes for use in the diagnosis of, treatment of or vaccination against a disease in an individual. In one example, fluorescent dextran conjugates of HLA-A2-peptide complexes are shown to be able to detect antigen-specific T-cells in melanoma patients. [This abstract record is one of 24 records for this document necessitated by the large number of index entries required to fully index the document and publication system constraints.].

IT 320-67-2
RL: THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(in combination therapy with MHC-peptide multimer vaccines)

RN 320-67-2 CAPLUS

CN 1,3,5-Triazin-2(1H)-one, 4-amino-1-β-D-ribofuranosyl- (CA INDEX NAME)

Absolute stereochemistry.



L7 ANSWER 22 OF 28 CAPLUS COPYRIGHT 2011 ACS on STN

ED Entered STN: 20 Apr 2010

ACCESSION NUMBER: 2010:490023 CAPLUS

DOCUMENT NUMBER: 152:499366

TITLE: MHC multimers in cancer vaccines and immune monitoring

INVENTOR(S): Bris, Liselotte; Schoeller, Joergen; Pedersen, Henrik

PATENT ASSIGNEE(S): Dako Denmark A/S, Den.

SOURCE: PCT Int. Appl., 1228pp., MEGA TAN OF 21324005W
CODEN: PIXXD2

DOCUMENT TYPE: Patent

LANGUAGE: English

FAMILY ACC. NUM. COUNT: 24

PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2010037395	A2	20100408	WO 2009-XJ50255	20091001

W: 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

RW: 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, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG, BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM

PRIORITY APPLN. INFO.:

DK 2008-1382	A	20081001
US 2008-101878P	P	20081001
EP 2009-154516	A	20090306

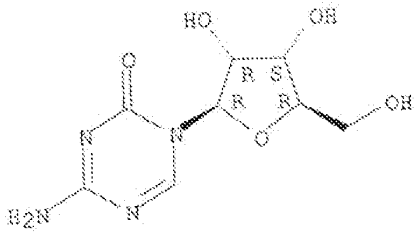
AB The authors disclose the preparation of multimeric MHC-peptide complexes for use in the diagnosis of, treatment of or vaccination against a disease in an individual. In one example, fluorescent dextran conjugates of

HLA-A2-peptide complexes are shown to be able to detect antigen-specific T-cells in melanoma patient [This abstract record is one 24 records for this document necessitated by the large number of index entries required to fully index the document and publication system constraints.].

IT 320-67-2
RL: THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(in combination therapy with MHC-peptide multimer vaccines)

RN 320-67-2 CAPLUS
CN 1,3,5-Triazin-2(1H)-one, 4-amino-1-β-D-ribofuranosyl- (CA INDEX NAME)

Absolute stereochemistry.



L7 ANSWER 23 OF 28 CAPLUS COPYRIGHT 2011 ACS on STN
ED Entered STN: 20 Apr 2010
ACCESSION NUMBER: 2010:490022 CAPLUS
DOCUMENT NUMBER: 152:452051
TITLE: MHC multimers in cancer vaccines and immune monitoring
INVENTOR(S): Brix, Liselotte; Schoeller, Joergen; Pedersen, Henrik
PATENT ASSIGNEE(S): Dako Denmark A/S, Den.
SOURCE: PCT Int. Appl., 1226pp., MEGA TAN OF 21324085W
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 24
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2010037395	A2	20100408	WO 2009-XT50258	20091001

W: 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
RW: 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, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG, BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM

PRIORITY APPLN. INFO.:

DK 2008-1382	A	20081001
US 2008-101878P	P	20081001
EP 2009-154516	A	20090306

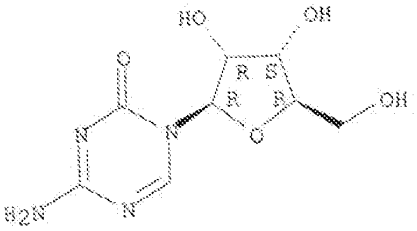
AB The authors disclose the preparation of multimeric MHC-peptide complexes for use in the diagnosis of, treatment of or vaccination against a disease in an individual. In one example, fluorescent dextran conjugates of HLA-A2-peptide complexes are shown to be able to detect antigen-specific T-cells in melanoma patients. [This abstract record is one of 24 records for this document necessitated by the large number of index entries required to fully index the document and publication system constraints.].

IT 320-67-2
RL: THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(in combination therapy with MHC-peptide multimer vaccines)

RN 320-67-2 CAPLUS
CN 1,3,5-Triazin-2(1H)-one, 4-amino-1-β-D-ribofuranosyl- (CA INDEX

NAME)

Absolute stereochemistry.



L7 ANSWER 24 OF 28 CAPLUS COPYRIGHT 2011 ACS on STN
ED Entered STN: 20 Apr 2010
ACCESSION NUMBER: 2010:490021 CAPLUS
DOCUMENT NUMBER: 152:427624
TITLE: MHC multimers in cancer vaccines and immune monitoring
INVENTOR(S): Brix, Liselotte; Schoeller, Joergen; Pedersen, Henrik
PATENT ASSIGNEE(S): Dako Denmark A/S, Den.
SOURCE: PCT Int. Appl., 1228 pp., MEGA TAN OF 21324085W
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 24
PATENT INFORMATION:

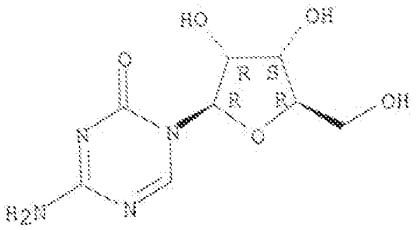
PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2010037395	A2	20100408	WO 2009-XP50255	20091001
W:	AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, 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			
RW:	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, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG, BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM			
PRIORITY APPLN. INFO.:			DK 2008-1382	A 20081001
			US 2008-101878P	P 20081001
			EP 2009-154516	A 20090306

AB The authors disclose the preparation of multimeric MHC-peptide complexes for use in the diagnosis of, treatment of or vaccination against a disease in an individual. In one example, fluorescent dextran conjugates of HLA-A2-peptide complexes are shown to be able to detect antigen-specific T-cells in melanoma patients. [This abstract record is one of 24 records for this document necessitated by the large number of index entries required to fully index the document and publication system constraints.].

IT 320-67-2
RL: THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(in combination therapy with MHC-peptide multimer vaccines)

RN 320-67-2 CAPLUS
CN 1,3,5-Triazin-2(1H)-one, 4-amino-1-β-D-ribofuranosyl- (CA INDEX NAME)

Absolute stereochemistry.



L7 ANSWER 25 OF 28 CAPLUS COPYRIGHT 2011 ACS on STN
ED Entered STN: 20 Apr 2010
ACCESSION NUMBER: 2010:490020 CAPLUS
DOCUMENT NUMBER: 152:452050
TITLE: MHC multimers in cancer vaccines and immune monitoring
INVENTOR(S): Brix, Liselotte; Schoeller, Joergen; Pedersen, Henrik
PATENT ASSIGNEE(S): Dako Denmark A/S, Den.
SOURCE: PCT Int. Appl., 1228pp., MEGA TAN OF 21324085W
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 24
PATENT INFORMATION:

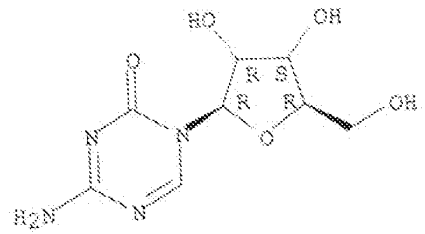
PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2010037395	A2	20100408	WO 2009-XK50255	20091001
W: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, 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				
RW: 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, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG, BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM				
PRIORITY APPLN. INFO.:			DK 2008-1382	A 20081001
			US 2008-101878P	P 20081001
			EP 2009-154516	A 20090306

AB The authors disclose the preparation of multimeric MHC-peptide complexes for use in the diagnosis of, treatment of or vaccination against a disease in an individual. In one example, fluorescent dextran conjugates of HLA-A2-peptide complexes are shown to be able to detect antigen-specific T-cells in melanoma patients. [This abstract record is one of 24 records for this document necessitated by the large number of index entries required to fully index the document and publication system constraints.].

IT 320-67-2
BL: THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(in combination therapy with MHC-peptide multimer vaccines)

RN 320-67-2 CAPLUS
CN 1,3,5-Triazin-2(1H)-one, 4-amino-1-β-D-ribofuranosyl- (CA INDEX NAME)

Absolute stereochemistry.



L7 ANSWER 26 OF 28 CAPLUS COPYRIGHT 2011 ACS on STN
ED Entered STN: 08 Apr 2010
ACCESSION NUMBER: 2010:434752 CAPLUS
DOCUMENT NUMBER: 152:427622
TITLE: MHC multimers in cancer vaccines and immune monitoring
INVENTOR(S): Brix, Liselotte; Schoeller, Joergen; Pedersen, Henrik
PATENT ASSIGNEE(S): Dako Denmark A/S, Den.
SOURCE: PCT Int. Appl., 1228pp.
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 24
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2010037395	A2	20100408	WO 2009-DK50255	20091001
WO 2010037395	A3	20100520		
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RW: 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, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG, BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM, AP, EA, EP, OA				
EP 2337795	A2	20110629	EP 2009-744607	20091001
R: AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LI, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR, AL, BA, RS				

PRIORITY APPLN. INFO.:

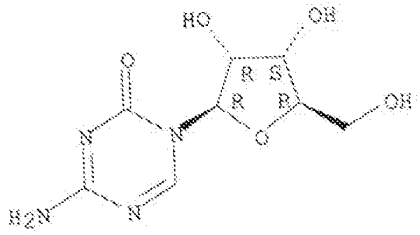
DK 2008-1382	A 20081001
US 2008-101878P	P 20081001
EP 2009-154516	A 20090306
WO 2009-DK50255	W 20091001

AB The authors disclose the preparation of multimeric MHC-peptide complexes for use in the diagnosis of, treatment of or vaccination against a disease in an individual. In one example, fluorescent dextran conjugates of HLA-A2-peptide complexes are shown to be able to detect antigen-specific T-cells in melanoma patients. [This abstract record is one of 24 records for this document necessitated by the large number of index entries required to fully index the document and publication system constraints.].

IT 320-67-2, Azacytidine
RL: THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(in combination therapy with MHC-peptide multimer vaccines)

RN 320-67-2 CAPLUS
CN 1,3,5-Triazin-2(1H)-one, 4-amino-1-β-D-ribofuranosyl- (CA INDEX NAME)

Absolute stereochemistry.



L7 ANSWER 27 OF 28 CAPLUS COPYRIGHT 2011 ACS on STN
ED Entered STN: 08 Apr 2010

ACCESSION NUMBER: 2010:437755 CAPLUS
DOCUMENT NUMBER: 152:452 3
TITLE: Molecular vaccines for infectious disease
INVENTOR(S): Schoeller, Joergen; Pedersen, Henrik; Brix, Liselotte
PATENT ASSIGNEE(S): Dako Denmark A/S, Den.
SOURCE: PCT Int. Appl., 716pp.
CODEN: PIXXD2

DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 3
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2010037402	A1	20100408	WO 2009-DK50262	20091002
W: 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				
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WO 2010037402	A1	20100408	WO 2009-XA50262	20091002
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WO 2010037402	A1	20100408	WO 2009-XB50262	20091002
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PRIORITY APPLN. INFO.:		
DK 2008-1384	A	20081002
US 2008-102126P	P	20081002
WO 2009-DK50262	W	20091002

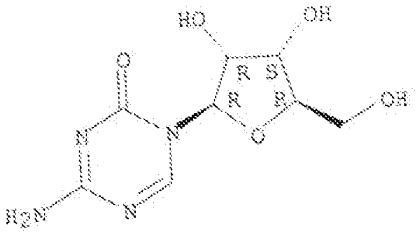
AB The authors disclose the construction of pharmamers (i.e., vaccine components characterized by their multimerization domain and the attached biol. active mols.) and their use in preparation of vaccines that contains the pharmamers alone or in combination with other mols. The individual mols. of the construct can be bound to each other or the multimerization domain(s) by covalent or non-covalent bonds, directly or via linkers. In one example, a pharmamer vaccine targeting immunodeficiency virus is constructed of streptavidin-modified dextran for multimerization of biotinylated MHC class I and class II complexes loaded with peptides from HIV Gag and Env and SIV Nef. [This abstract record is one of three records for this document necessitated by the large number of index entries required to fully index the document and publication system constraints.].

IT 320-67-2, Azacitidine

RL: THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(in combination therapy ch MHC-based vaccine pharmamers

RN 320-67-2 CAPLUS
CN 1,3,5-Triazin-2(1H)-one, 4-amino-1-β-D-ribofuranosyl- (CA INDEX NAME)

Absolute stereochemistry.



REFERENCE COUNT: 9 THERE ARE 9 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

L7 ANSWER 28 OF 28 CAPLUS COPYRIGHT 2011 ACS on STN
ED Entered STN: 20 Nov 2009
ACCESSION NUMBER: 2009:1436491 CAPLUS
DOCUMENT NUMBER: 151:537097
TITLE: Oral formulations of cytidine analogs for gastric release and methods of use thereof
INVENTOR(S): Etter, Jeffrey B.; Lai, Mei; Backstrom, Jay Thomas
PATENT ASSIGNEE(S): Celgene Corporation, USA
SOURCE: PCT Int. Appl., 130pp.
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 1
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2009139888	A1	20091119	WO 2009-US2999	20090514
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RW: 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, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG, BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM				
AU 2009246926	A1	20091119	AU 2009-246926	20090514
US 20090286752	A1	20091119	US 2009-466213	20090514
KR 20110015629	A	20110216	KR 2010-7028070	20090514
EP 2299984	A1	20110330	EP 2009-746975	20090514
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CN 102099018	A	20110615	CN 2009-80127929	20090514
AR 71808	A1	20100714	AR 2009-101759	20090515
MX 2010012470	A	20101202	MX 2010-12470	20101112
			US 2008-53609P P	20080515
			US 2008-201145P P	20081205
			US 2009-157875P P	20090305
			WO 2009-US2999 W	20090514

PRIORITY APPLN. INFO.:

ASSIGNMENT HISTORY FOR US PATENT AVAILABLE IN LSUS DISPLAY FORMAT

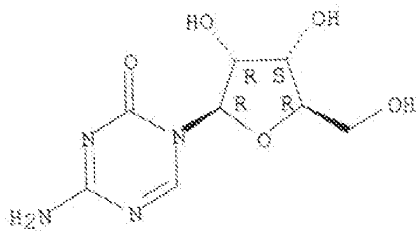
AB The present disclosure provides pharmaceutical compns. comprising cytidine analogs for oral administration, wherein the compns. release the cytidine

analog substantially in the stomach. Also provided are methods of treating diseases and disorders including cancer, disorders related to abnormal cell proliferation, hematol. disorders, and immune disorders, among others, using the oral formulations provided herein. Thus, azacytidine non-enteric coated tablets comprised (in wt%): azacytidine 60.0, mannitol 129.6, silicified microcryst. cellulose 90.0, crospovidone 9.0, magnesium stearate 5.4, vitamin E TPGS 6.0, hydroxypropyl cellulose 12.0.

IT 320-67-2, 5-Azacytidine
RL: BSU (Biological study, unclassified); PAC (Pharmacological activity); PKT (Pharmacokinetics); THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(oral formulations of cytidine analogs for gastric release and methods of use thereof)

RN 320-67-2 CAPLUS
CN 1,3,5-Triazin-2(1H)-one, 4-amino-1-β-D-ribofuranosyl- (CA INDEX NAME)

Absolute stereochemistry.



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REFERENCE COUNT: 4 THERE ARE 4 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

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L3            26 S L1 SSS FULL

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L5            52 S L4 AND MAGNESIUM
L6            32 S L5 AND STOMACH
L7            28 S L6 AND MANNITOL

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L6            32 S L5 AND STOMACH
L7            28 S L6 AND MANNITOL

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FULL ESTIMATED COST                              178.64     376.75

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CA SUBSCRIBER PRICE                              -24.36     -24.36

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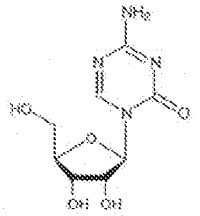
water, DMF, DMSO. Max stability of aq solns is at pH 4-8. Moderately sol in methanol. LD_{50} in mice, rats, and chickens: >10000 mg/kg orally (Am. Cyanamid, company literature).

α-Avoparcin. [73957-86-5] $C_{86}H_{102}ClN_9O_{36}$. $[\alpha]_D^{25}$ −96 ±2° (c = 0.62 in 0.1*N* HCl). uv max (0.1*N* HCl): 280 nm ($E_{1cm}^{1\%}$ 42.0).

β-Avoparcin. [73957-87-6] $C_{86}H_{101}Cl_2N_9O_{36}$. $[\alpha]_D^{25}$ −102 ±2° (c = 0.65 in 0.1*N* HCl). uv max (0.1*N* HCl): 280 nm ($E_{1cm}^{1\%}$ 44.0).

THERAPCAT (VET): Antibacterial; growth promotant.

890. Azacitidine. [320-67-2] 4-Amino-1-β-D-ribofuranosyl-1,3,5-triazin-2(1*H*)-one; 5-azacytidine; 5-AzaC; ladakamycin; U-18496; NSC-102816; Mylosar (formerly); Vidaza. $C_8H_{12}N_4O_5$; mol wt 244.20. C 39.35%, H 4.95%, N 22.94%, O 32.76%. DNA methylation inhibitor; analog of the pyrimidine nucleoside, cytidine, *q.v.* Chemical synthesis: A. Piskala, F. Sorm, *Collect. Czech. Chem. Commun.* **29**, 2060 (1964); M. W. Winkley, R. K. Robins, *J. Org. Chem.* **35**, 491 (1970). Production by fermentation of *Streptoverticillium ladakanus* and activity: L. J. Hanka *et al.*, *Antimicrob. Agents Chemother.* **1966**, 619; M. E. Bergy, R. R. Herr, *ibid.* 625. HPLC determn in pharmaceutical preps: L. D. Kissinger, N. L. Stemm, *J. Chromatogr.* **353**, 309 (1986). Toxicology study: P. E. Palm, C. J. Kensler, *U.S. Clearinghouse Fed. Sci. Tech. Inform.* (PB-194791, 1970) 191 pp.; *C.A.* **75**, 33704j (1971). Review of clinical experience in acute nonlymphocytic leukemia: A. B. Glover *et al.*, *Cancer Treat. Rep.* **71**, 737-746 (1987); of mechanism of action: A. B. Glover, B. Leyland-Jones, *ibid.* 959-964. Review of carcinogenic risk: *IARC Monographs* **50**, 47-63 (1990). Clinical efficacy in β-thalassemia: C. H. Lowrey, A. W. Nienhuis, *N. Engl. J. Med.* **329**, 845 (1993); in myelodysplastic syndrome: L. R. Silverman *et al.*, *J. Clin. Oncol.* **20**, 2429 (2002); A. B. Kornblith *et al.*, *ibid.* 2441.

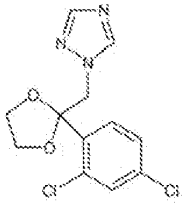


Crystals from aq ethanol, mp 235-237° (dec). $[\alpha]_D^{20}$ +22.4° (c = 1 in water). uv max (water): 241 nm (ε 8767); (0.01*N* HCl): 249 nm (ε 3077); (0.01*N* KOH): 223 nm (ε 24209). Solv (mg/ml): 40 warm water, 14 cold water, 28 0.1*N* HCl, 43 0.1*N* NaOH, 52.7 DMSO, 1 acetone, 1 chloroform, 1 hexane. LD_{50} in mice (mg/kg): 115.9 i.p.; 572.3 orally (Palm, Kensler).

Caution: This substance is reasonably anticipated to be a human carcinogen: *Report on Carcinogens, Eleventh Edition* (PB2005-104914, 2004) p III-24.

THERAPCAT: Antineoplastic.

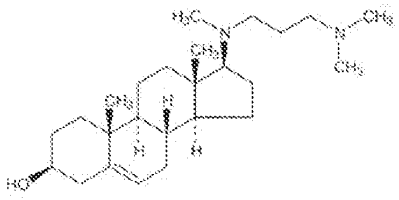
891. Azaconazole. [60207-31-0] 1-[[2-(2,4-Dichlorophenyl)-1,3-dioxolan-2-yl]methyl]-1*H*-1,2,4-triazole; R-28644; Redeswod; Safetray. $C_{12}H_{11}Cl_2N_3O_2$; mol wt 300.14. C 48.02%, H 3.69%, Cl 23.62%, N 14.00%, O 10.66%. Sterol demethylation inhibitor. Prepn: G. Van Reet *et al.*, **DE 2551668**; *eidem*, **US 4079062** (1975, 1978 both to Janssen). Wood fungicide for mushroom cultivation: L. Van Leemput *et al.*, *Med. Fac. Landbouww. Rijksuniv. Gent* **52**, 703 (1987); A. Eicker, E. Strydom, *Bot. Bull. Acad. Sinica* **31**, 51 (1990). Use as a preservative for composite wood products: E. L. Schmidt, R. O. Gertjejansen, *Forest Prod. J.* **38**, 19 (1988); A. Jeihoomi *et al.*, *Wood Fiber Sci.* **26**, 178 (1994).



Crystals from diisopropylether, mp 109.9°.

USE: Fungicide for cultivation on wood; preservative for composite wood products.

892. Azacosterol. [313-05-3] (3*β*,17*β*)-17-[[3-(Dimethylamino)propyl]methylamino]androst-5-en-3-ol; *N*-methyl-*N*-[3-(dimethylamino)propyl]-17*β*-aminoandrost-5-en-3*β*-ol; 20,25-diazacholesterol; diazasterol. $C_{25}H_{48}N_2O$; mol wt 388.63. C 77.26%, H 11.41%, N 7.21%, O 4.12%. Prepn: Counsell *et al.*, *J. Med. Pharm. Chem.* **5**, 1224 (1962); Counsell, Klimstra, **US 3084158** (1963 to Searle).

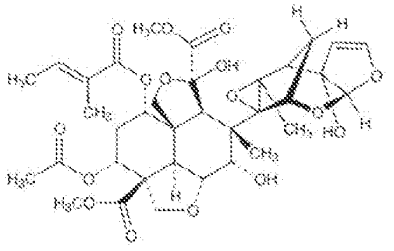


Crystals from acetone + methanol, mp 146-148°. $[\alpha]_D$ −54.5°.

Dihydrochloride. [1249-84-9] SC-12937; Ornitrol. $C_{25}H_{48}N_2.2HCl$; mol wt 461.55. $[\alpha]_D$ −32°.

THERAPCAT (VET): Avian chemosterilant.

893. Azadirachtin. [11141-17-6] $C_{35}H_{44}O_{16}$; mol wt 720.71. C 58.33%, H 6.15%, O 35.52%. A tetranortriterpinoid isolated from the seeds of the neem tree, *Azadirachta indica* A. Juss. (*Melia azadirachta* L.), *Meliaceae*, and the chinaberry tree, *M. azedarach* L. Highly active insect feeding deterrent and growth regulator. Isoln from *A. indica* and identification as feeding inhibitor in locusts: J. H. Butterworth, E. D. Morgan, *Chem. Commun.* **1968**, 23; from *M. azedarach*: E. D. Morgan, M. D. Thornton, *Phytochemistry* **12**, 391 (1973). Partial synthesis: D. Pfleiger *et al.*, *Tetrahedron Lett.* **28**, 1519 (1987). Structural studies: J. B. Butterworth *et al.*, *J. Chem. Soc. Perkin Trans. 2* **1972**, 2445. ^1H- and ^{13}C-NMR data and structure: P. R. Zanno *et al.*, *J. Am. Chem. Soc.* **97**, 1975 (1975); K. Nakanishi in *Recent Advances in Phytochemistry* **vol. 9**, V. C. Runeckles, Ed. (Plenum Press, New York, 1975) pp 283-298. Revised structure: W. Kraus *et al.*, *Tetrahedron Lett.* **26**, 6435 (1985); H. B. Broughton *et al.*, *Chem. Commun.* **1986**, 46. Isoln by HPLC: E. C. Uebel *et al.*, *J. Liq. Chromatogr.* **2**, 875 (1979); J. D. Warthen, Jr. *et al.*, *ibid.* **7**, 591 (1984). Antifeedant activity in locusts: J. S. Gill, C. T. Lewis, *Nature* **232**, 402 (1971); in fall army worms, cotton bollworms: J. A. Klocke, I. Kubo, *Entomol. Exp. Appl.* **32**, 299 (1982). Insect ecdysis and growth inhibition: H. Rembold, K. P. Siebes, *Z. Naturforsch.* **36C**, 466 (1981); I. Kubo, J. A. Klocke, *Agric. Biol. Chem.* **46**, 1951 (1982); K. P. Siebes, H. Rembold, *J. Insect Physiol.* **29**, 523 (1983). Series of articles on chemistry and activity: *Natural Pesticides from the Neem Tree*, Proc. 1st Int. Neem Conf., 1980, H. Schmutterer *et al.*, Eds. (German Agency for Technical Cooperation, Eschborn, 1981) 291 pp.



Microcrystalline powder from carbon tetrachloride, mp 154-158°. $[\alpha]_D$ −53° (c = 0.5 in $CHCl_3$). uv max (methanol): 217 nm (ε 9160).

USE: Experimentally as insect control agent.

894. Azalenidin. [68049-83-2] 2-(2,4-Dichloro-5-(2-propynyloxy)phenyl)-5,6,7,8-tetrahydro-1,2,4-triazolo[4,3-*a*]pyridin-3(2*H*)-one; DPX-R6447; Milestone; Evolus. $C_{15}H_{13}Cl_2N_3O_2$; mol

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Inhibition of P-Glycoprotein by D-α-Tocopheryl Polyethylene Glycol 1000 Succinate (TPGS)

Jay M. Dintaman[1] and Jeffrey A. Silverman[1,2]

Received March 5, 1999; accepted June 30, 1999

Purpose. To investigate whether d-α-tocopheryl polyethylene glycol 1000 succinate (TPGS) functions as an inhibitor of P-glycoprotein (P-gp), the multidrug resistance transporter.

Methods. Two assays were used to measure the function of TPGS on P-gp function. First, we examined the ability of TPGS to modulate the cytotoxicity of established, cytotoxic, P-glycoprotein substrates. Parental NIH 3T3 cells and NIH 3T3 cells transfected with the human MDR1 cDNA (G185) were exposed to doxorubicin, paclitaxel, colchicine, vinblastine and 5-fluorouracil (5FU) in the presence or absence of TPGS. Cytotoxicity was assessed with the MTT assay. Second, polarized transport of the P-gp substrates rhodamine 123 (R123), paclitaxel and vinblastine was measured using the human intestinal HCT-8 and Caco-2 cell lines grown in Transwell dishes. Drug flux was measured by liquid scintillation counting or fluorescence spectroscopy of the media.

Results. G185 cells were 27–135 fold more resistant to the cytotoxic drugs doxorubicin, vinblastine, colchicine and paclitaxel than the parental NIH 3T3 cells. In contrast 5FU, which is not a P-gp substrate, is equally cytotoxic to parental and G185 cells. Co-administration of TPGS enhanced the cytotoxicity of doxorubicin, vinblastine, paclitaxel, and colchicine in the G185 cells to levels comparable to the parental cells. TPGS did not increase the cytotoxicity of 5FU in the G185 cells. Using a polarized epithelial cell transport assay, TPGS blocked P-gp mediated transport of R123 and paclitaxel in a dose responsive manner.

Conclusions. These data demonstrate that TPGS acts as a reversal agent for P-glycoprotein mediated multidrug resistance and inhibits P-gp mediated drug transport. These results suggest that enhanced oral bioavailability of drugs co-administered with TPGS may, in part, be due to inhibition of P-glycoprotein in the intestine.

KEY WORDS: P-glycoprotein; TPGS; drug transport; bioavailability.

INTRODUCTION

The multidrug transporter, P-glycoprotein (P-gp), is a 170 kDa membrane protein which functions as an ATP-dependent drug efflux pump. One activity of this protein is to lower the intracellular concentration of drugs thereby reducing the cytotoxic activity of anticancer drugs. Increased expression of this protein has been observed in human tumors and is often associated with failure of chemotherapy due to drug resistance (1–5). P-gp removes a large number of chemically unrelated drugs extending over many therapeutic indications such as anticancer drugs, steroids, antihistamines, antibiotics, calcium channel blockers and anti-HIV peptidomimetics (2,4,5).

The P-gp drug transporter is encoded by one gene, MDR1, in humans whereas in rodents two genes, *mdr1a* and *mdr1b* encode highly similar drug transporters (6,7). P-gp is primarily expressed on the luminal surface of epithelial cells from several tissues including the intestine, liver, kidney, and the endothelial cells comprising the blood-brain and blood-testes barriers (8–10). The ability of this protein to export toxic compounds combined with this localization led to the hypothesis that a physiological function of the MDR1 encoded P-gp may be as a protective barrier or export mechanism for xenobiotics. Indeed, recent investigations with knockout mice in which the *mdr1a* gene was disrupted have confirmed such a protective role for P-gp (11–14). Exposure of *mdr1a* deficient mice to vinblastine or ivermectin results in significantly higher tissue and plasma levels compared to wild-type animals. Moreover, these compounds are toxic in the knockout mice at doses which are innocuous to heterozygous and wild-type mice. These experiments further suggested a role for P-gp in the blood brain barrier since the ivermectin accumulated in the brain of the *mdr1a* deficient animals but not animals with an intact *mdr1a* gene. The knockout mice displayed ivermectin toxicity at doses 50 to 100 fold less than wild-type mice.

Additional data have supported a role of P-gp in the intestine as both a barrier to absorption as well as a mechanism of disposition of drugs such as vinblastine, etoposide, paclitaxel and digoxin. For example, Su and Huang observed that inhibition of P-gp increased bioavailability of digoxin by increasing absorption as well as reducing excretion (15). A similar phenomenon was observed with etoposide (16). P-glycoprotein has recently been suggested to be critical in oral drug absorption (17–19). In concert with the drug metabolizing enzyme CYP3A, P-gp may limit oral drug bioavailability in the gut by controlling drug transport from the intestinal lumen and by affecting access to CYP3A (19).

Vitamin E TPGS, d-α-tocopheryl polyethylene glycol 1000 succinate, is a derivative of vitamin E consisting of a hydrophilic polar head group (tocopherol succinate) and a lipophilic alkyl tail (polyethylene glycol) resulting in amphiphillic properties (Eastman Kodak, technical bulletin EFC-226). TPGS has a relatively low critical micelle concentration, 0.02 wt%, and acts to solubilize lipophilic compounds. Bordreaux et al. reported a two-fold increase in cyclosporine CsA area under the plasma concentration-time-curve (AUC) when co-administered with LiquiE, a glycerol and water solution of TPGS (20). Sokol et al. similarly observed increases up to 71% in CsA AUC in subjects who received concomitant TPGS (21). Both Sokol and Bordreaux suggested that the increased drug absorption was due to enhanced micelle formation, resulting in improved CsA solubilization. Chang *et al.* later reported a 61% increase in CsA AUC when dosed with 20–25% of the TPGS previously used in the Sokol or Bordreaux studies (22). Chang *et al.* also suggested that TPGS may interact with P-gp in the intestine to increase CsA absorption.

In the current investigation we examine the effect of TPGS on P-gp mediated drug resistance and transport of established P-gp substrates. If this agent functions as a P-gp reversal agent then perhaps its effect on drug absorption is, in part, mediated by inhibition of active drug efflux in the intestine. Our data show TPGS to be an effective inhibitor of P-gp mediated drug

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ABBREVIATIONS: P-gp, P-glycoprotein; R 123, Rhodamine 123; CsA, Cyclosporine A; TPGS, d-α-tocopheryl polyethylene glycol 1000 succinate.

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resistance and transport at concentrations well below the reported critical micelle concentration and suggests that its reversal activity is due to an effect on transport activity.

MATERIALS AND METHODS

Cell Culture

The NIH3T3 Swiss mouse embryo cell line was obtained from American Type Culture Collection (ATCC, Rockville, MD) and was grown in Dulbecco's Modified Eagles Medium (Biowhittaker, Walkersville, MD) supplemented with 4.5 g/L glucose, 10% fetal bovine serum (Hyclone Laboratories, Logan, UT), 2 mM L-glutamine (Advanced Biotechnologies Incorporated (ABI), Columbia, MD), and 0.01 mg/ml gentamicin (ABI). The drug resistant, NIH-MDR-G185, cell line, derived by transfection of the human MDR1 gene into NIH3T3 cells (23), was obtained from M. M. Gottesman (NCI, NIH) and was maintained in similar medium supplemented with 60 ng/ml of colchicine (Sigma, St. Louis, MO). HCT-8 cells (ATCC), isolated from a human ileocecal adenocarcinoma cell line, were grown in RPMI-1640 medium (Biowhittaker) supplemented with 10% horse serum (Biowhittaker), 1 mM sodium pyruvate (Gibco BRL, Grand Island NY) and 0.01 mg/ml gentamicin. Caco-2 cells (ATCC), derived from a human colonic adenocarcinoma, were grown in Eagle's MEM (Biowhittaker) supplemented with 10% fetal bovine serum, and 0.01 mg/ml gentamicin. All cells were maintained in a humidified atmosphere with 5% CO_2 at 37°C.

Cytotoxicity Assay

Cells were plated at a density of 2.5–3.0 × 10^3 cells/well in 96-well microtiter plates (PGC, Gaithersburg, MD) and were exposed to 1–5000 nM of doxorubicin, vinblastine, colchicine, paclitaxel, 0.1–25 nM 5-fluorouracil (Sigma) and 0.001–.005% TPGS (Eastman, Kingsport, TN) for 72 hours. To ensure solubilization of the TPGS, a 1% solution of TPGS in ethanol was prepared fresh for each experiment and diluted further in cell culture medium to the indicated concentrations. Cell viability was determined with the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium, Sigma) assay as previously described (24,25) and the absorbance was measured with a Dynex MRX Microplate Reader (Chantilly, VA) at 570 nm. This assay is based on the reduction of MTT by mitochondria in viable cells to water insoluble formazan. The data presented are the mean ±SD of at least 3 independent experiments, each performed in quadruplicate.

Rhodamine 123 Transport

Rhodamine 123 (R123; Sigma) transport was examined as previously described (26,27) using both HCT-8 and Caco-2 cells. Briefly, cells were grown in 6 well Corning Transwell dishes (HCT-8) or collagen coated Transwell dishes (Caco-2) until a tight monolayer was formed as measured by transepithelial electrical resistance or lucifer yellow impermeability. The integrity of the monolayers following the transport experiments was similarly evaluated. Typical TEER values were > 300 Ohms/cm^2. R123 was added at a final concentration of 13 μM to the basal or apical compartments and 200 μl samples were taken at the indicated times from the opposite chamber. TPGS was added as an inhibitor to both compartments. Fluorescence of R123 in the media samples was measured using a Biotek FL500 Fluorescence Plate Reader (Winooski, VT) with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. All experiments were performed in triplicate; the data presented are the mean ±SD and are representative of multiple experiments.

Paclitaxel, Vinblastine and Cyclosporine Transport

Inhibition of [^{3}H] paclitaxel (Moravek Biochemical, Brea, CA), [^{3}H] vinblastine (Amersham, Arlington Heights, IL), and [^{3}H] cyclosporine (CsA; Amersham) efflux by TPGS was examined in a manner similar to R123. The transported drug, 0.1 μM (0.25 μCi/ml), was added to either the basal or apical compartment and 200 μl aliquots were taken at the indicated times from the opposite chamber. Radioactivity was measured by liquid scintillation counting.

Western Blot Analysis

Western blot analysis was performed as previously described 28. Briefly, crude cell membranes were isolated by lysing the cells in 10 mM Tris-HCl, pH 7.5; 10 mM NaCl; 1 mM $MgCl_2$ supplemented with pepstatin (1.5 μg/ml), leupeptin (1.5 μg/ml) and 0.2 mM pefabloc. Cells were homogenized with 20 strokes of Dounce "B" (tight) pestle (Wheaton, Millville, NJ), nuclei and cell debris were removed by centrifugation for 10 minutes at 400 × g. The supernatants were then centrifuged at 100,000 × g for 30 minutes at 4°C and the pellets were resuspended in lysis buffer and stored at −80°C. 20 μg samples were fractionated in 8% polyacrylamide-SDS gel and transferred to 0.45 μm nitrocellulose membrane. The membranes were blocked in PBS-T (0.1% Tween-20 in PBS) containing 5% skim milk for 1 hour and then probed with 1 μg/ml of C219 antibody (Signet Laboratories, Dedham, MA) in PBS overnight. The membranes were visualized by enhanced chemiluminescence according to the manufacturer's instructions (Pierce, Rockford, IL).

RESULTS

Western Blot Analysis

We first measured the relative levels of P-gp expression in the NIH3T3 and G185 cell lines by western blot analysis using the C219 antibody, which recognizes all P-gp isoforms (29). Consistent with previous data, high P-gp expression was observed in the G185 cells relative to that in the parental NIH 3T3 cells (Fig. 1). We also examined P-gp expression in two human intestinal carcinoma cell lines, Caco-2 and HCT-8 which have been previously used for investigation of drug transport and to have polarized expression of P-gp (26). We observed that each of these intestinal cell lines have moderate P-gp expression albeit lower than the G185 cells (Fig. 1).

Cytotoxicity Experiments

The interaction of TPGS with P-gp was initially examined with cytotoxicity assays using parental NIH3T3 and MDR1 transfected G185 cells to cytotoxic anticancer drugs. Consistent with previous reports (23,30), G185 cells were more resistant

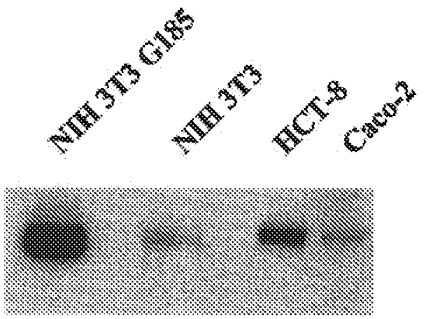

Fig. 1. Western blot analysis of P-glycoprotein expression. Twenty micrograms of total cell membrane proteins were separated by SDS-PAGE, transferred to PVDF filters which were subsequently probed with the C219 antibody and visualized using chemiluminescence as described in Materials and Methods. Lane 1, NIH-3T3 G185; lane 2, NIH-3T3; lane 3, HCT-8; lane 4, Caco-2.

Table 1. EC_{50} in NIH3T3 and NIH3T3-G185 Cells

	NIH3T3	NIH3T3-G185	NIH3T3-G185 (1 μM Verapamil)	NIH3T3-G185 (5 μM CsA)	NIH3T3-G185 (.0025% TPGS)
Doxorubicin	35	950	35	35	35
Vinblastine	2	270	20	6	40
Paclitaxel	60	>5000	100	40	1070
Colchicine	30	1000	ND	100	45

Note: NIH 3T3 and G185 cells were treated with 0–5000 nM doxorubicin, vinblastine, paclitaxel or colchicine in the absence or presence of 1 μM verapamil, 5 μM CsA or 0.0025% TPGS. The concentration of the drug that reduces cell viability by 50% (EC_{50}) was determined using the MTT cytotoxicity assay as described in Materials and Methods. Each experiment was performed in quadruplicate and repeated in at least 3 independent experiments. ND, not determined.

to doxorubicin, paclitaxel, vinblastine and colchicine compared to parental NIH3T3 cells (Fig. 2). EC_{50} values were 27 to 135 fold higher in G185 cells relative to the parental NIH3T3 cells (Table 1). Established P-gp reversal agents, such as cyclosporine A (CsA) and verapamil, reduced the resistance to doxorubicin cytotoxicity in G185 cells to levels comparable to parental NIH3T3 cells (Fig. 3). The reversal effect of CsA on doxorubicin, vinblastine, taxol and colchicine mediated toxicity in parental NIH3T3 cells was modest as previously reported. This is consistent with their low level of P-gp expression (data not shown, (27,30). Co-administration of CsA or verapamil caused a similar reversal of G185 resistance to vinblastine, paclitaxel, and colchicine (Table 1 and data not shown).

The effect of TPGS on P-gp mediated drug resistance was investigated by treating G185 cells with doxorubicin, vinblastine, paclitaxel, and colchicine concomitantly with varying doses of TPGS. The presence of TPGS increased drug sensitivity of the G185 cells to doxorubicin in a dose dependent manner

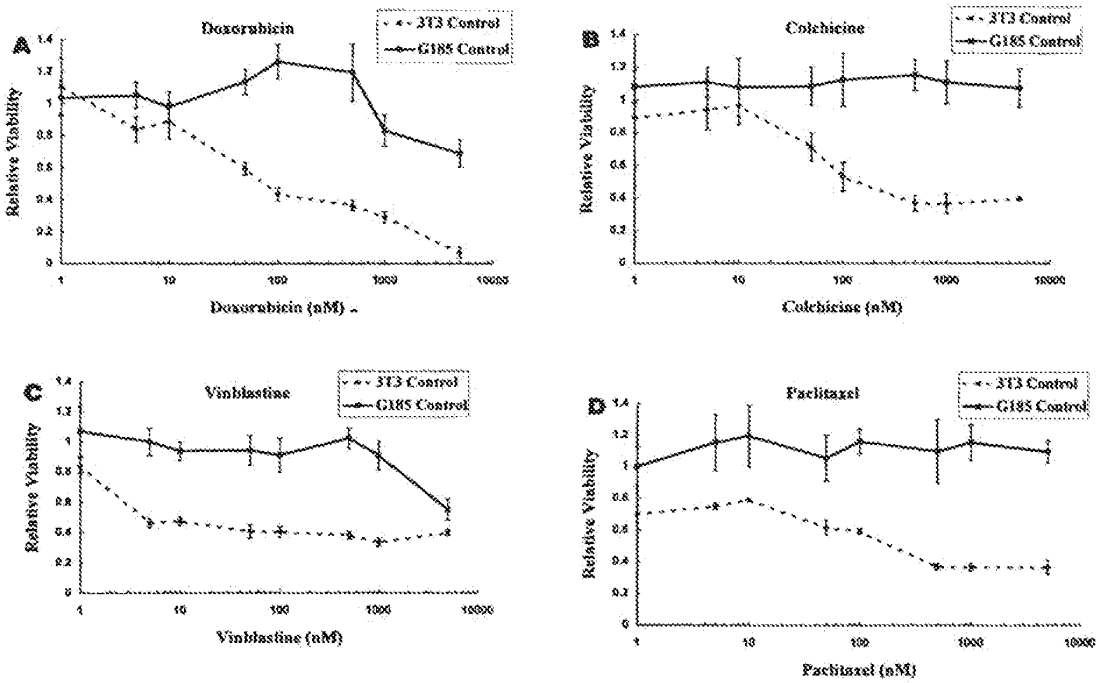

Fig. 2. Cytotoxicity of doxorubicin (A), colchicine (B), vinblastine (C), and taxol (D) in parental NIH-3T3 cells (circles), and MDR1-transfected NIH-3T3 G185 cells (squares). Cells were treated with the indicated concentrations of drugs and the viability was measured by the MTT assay as described in Materials and Methods. Data are expressed relative to untreated control cells. Each experiment was performed in quadruplicate and the data presented represent the mean ± SD of four independent experiments.

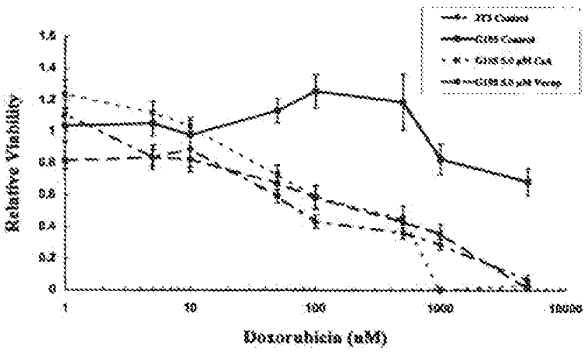

Fig. 3. Effect of CsA and Verapamil on the cytotoxicity of doxorubicin. Parental NIH-3T3 (diamonds) and G185 (squares) cells were exposed to the indicated concentrations of doxorubicin in the absence or presence of CsA, 5.0 μM, or verapamil, 5.0 μM.. Data are expressed relative to untreated control cells. Each experiment was performed in quadruplicate and the data presented represent the mean ± SD of four independent experiments.

(Fig. 4). Treatment of the drug resistant G185 cells with TPGS lowered the EC_{50} concentrations for doxorubicin, vinblastine, paclitaxel and colchicine (Table 1). TPGS, 0.0025%, sensitized the G185 cells to all four of these P-gp substrate cytotoxic drugs to levels comparable to the parental NIH 3T3 cells. The highest dose of TPGS, 0.005%, resulted in decreased viability of both NIH3T3 and G185 cells and is likely due to toxicity associated with the high concentration of TPGS. At concentrations below 0.005% TPGS itself did not affect cell viability. These data suggest that TPGS modulates drug resistance by inhibiting P-gp activity in cells which over-express the MDR1 gene.

5-Fluorouracil Cytotoxicity

Treatment of parental NIH 3T3 and G185 cells with 5-fluorouracil (5FU), a chemotherapeutic agent not transported by P-gp, results in a similar level of cytotoxicity in both cell lines (Fig. 5A) (31). Furthermore, co-incubation of 5FU with CsA had no effect on the cytotoxicity of 5FU in either G185 or NIH3T3 cells (Fig. 5B). Similarly, co-incubation of TPGS with 5FU did not increase the cytotoxicity of 5FU in either of these cell lines (Fig. 5C).

Rhodamine 123 Transport

The fluorescent dye R123, an established substrate of P-glycoprotein (32,33), was used to examine the ability of TPGS to block P-gp mediated transport. HCT-8 and Caco-2 cells have previously demonstrated directional transport of established P-gp substrates such as vinblastine, paclitaxel, CsA and R123 in the basolateral to apical direction (26,34–36). Expression of P-gp in these cells was confirmed by western blot analysis using the C219 antibody (Fig. 1). R123 was transported approximately 7 and 9 fold greater flux in the basolateral to apical direction in HCT-8 and Caco-2 cells, respectively (Fig. 6). Consistent with this transport being mediated by P-gp, R123 flux was inhibited approximately 80% by co-incubation with 5 μM CsA. Similarly, 0.001–0.0025% TPGS blocked the basolateral to apical transport of R123 in a dose responsive manner further suggesting that TPGS inhibits transport mediated by P-gp (Fig. 6).

Paclitaxel Transport

The ability of TPGS to inhibit P-gp was confirmed by measuring polarized transport of paclitaxel. [^{3}H] Paclitaxel is a good substrate for P-gp with approximately 14 and 40 fold greater transport from the basolateral to the apical compartment in HCT-8 and Caco-2 cells, respectively (Fig. 7). Addition of 5 μM CsA blocked the polarized flux of paclitaxel by 80–90%. Similarly, co-incubation with TPGS resulted in a dose dependent decrease in paclitaxel transport (Fig. 7). The IC_{50} of TPGS for inhibition of paclitaxel transport is approximately 0.001% (v/v) in HCT-8 cells and 0.005% in Caco-2 cells. Polarized

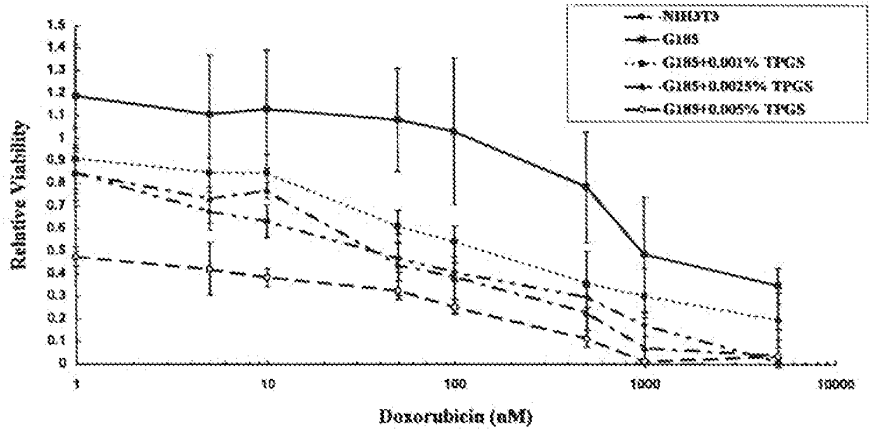

Fig. 4. TPGS reversal of P-gp mediated resistance to doxorubicin. Parental NIH-3T3 (diamonds) and G185 cells were exposed to the indicated concentrations of doxorubicin with 0% TPGS (squares), 0.001% TPGS (circles), 0.0025% TPGS (triangles) or 0.005% TPGS (open circles, ○). Data are expressed relative to untreated control cells. Each experiment was performed in quadruplicate and the data presented represent the mean ±SD of four independent experiments.

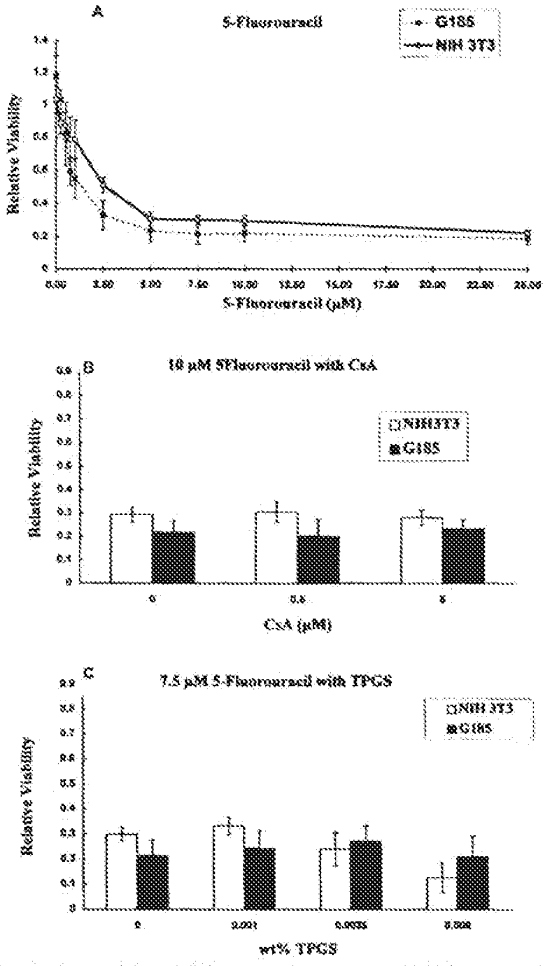

Fig. 5. Cytotoxicity of 5-fluorouracil to parental NIH-3T3, and drug resistent NIH-3T3 G185 cell lines in the presence and absence of CsA and TPGS. A. NIH-3T3 (diamonds) and NIH-3T3 G185 (squares) cells were exposed to the indicated concentrations of 5-fluorouracil. Viability was measured by the MTT assay as described in Materials and Methods. Data are expressed relative to untreated control cells. Each experiment was performed in quadruplicate and the data presented represent the mean ±SD of three independent experiments. B. Parental NIH-3T3 (open bars) and G185 (closed bars) cells were exposed to 10 μM of 5FU with the indicated concentrations of CsA. C. Parental NIH-3T3 (open bars) and G185 (closed bars) cells were exposed to 7.5 μM of 5FU and the indicated concentrations of TPGS.

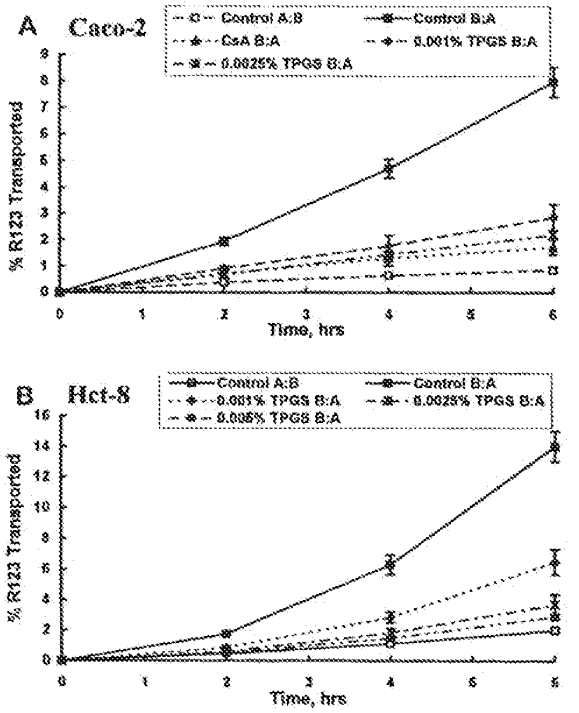

Fig. 6. Rhodamine 123 transport in Caco-2 and HCT-8 cells. Caco-2 (A) and HCT-8 (B) were grown on Transwell dishes as described in Materials and Methods. Rhodamine 123, 13 μM, was added to the apical or basolateral compartment in the absence or presence of CsA, 5 μM, or 0.0025, 0.005, 0.001% TPGS and media aliquots were taken from the opposite chamber at the indicated times. The data presented are the mean ±SD of triplicate wells and are representative of at least three independent experiments.

transport of [^{3}H] vinblastine and [^{3}H] CsA were also inhibited by addition of TPGS (data not shown). These data, combined with the cytotoxicity and R123 transport data suggest that TPGS is an effective P-gp reversal agent.

DISCUSSION

A major effort has been undertaken by many laboratories to identify inhibitors of P-glycoprotein to increase the efficacy of cancer treatment and to enhance the absorption of orally administered drugs. The data presented here support the hypothesis that TPGS functions as one such P-gp inhibitor. TPGS increased the sensitivity of P-gp expressing cells to several widely used cytotoxic drugs which are well established P-gp substrates. TPGS also effectively blocked polarized transport of R123 and paclitaxel in an epithelial cell transport assay. The reduction of directional transport provides strong evidence for TPGS functioning as an inhibitor of P-gp. Conversely, no effect was observed with 5FU, a cytotoxic drug not associated with P-gp mediated drug resistance or transport. 5FU is not transported by the P-gp pump thus, its cytotoxicity is unaffected by the addition of established P-gp inhibitors such as quinine, quinidine or verapamil (31,37). In the experiments presented here neither TPGS nor CsA impacted the cytotoxicity of 5FU in either the NIH 3T3 or G185 cells.

Previously it has been suggested that co-administration of TPGS with CsA enhanced absorption of the immunosuppressant due to micelle formation (21). Concentrations of TPGS administered in the current work are well below the critical micelle concentration, 0.02 wt% in water at 37°C, therefore it is unlikely that micelle formation is responsible for the observed effects. In fact, the IC_{50} required to inhibit R123 and paclitaxel transport across HCT-8 or Caco-2 cell monolayers is 20 fold less than the critical micelle concentration. Further, 0.001 wt% TPGS also significantly reversed the multidrug resistant phenotype of the NIH3T3-G185 cell line to doxorubicin, vinblastine, taxol

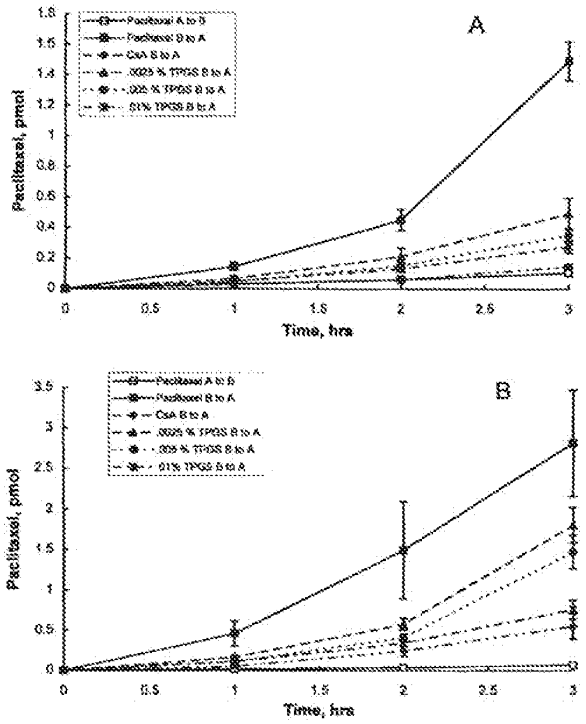

Fig. 7. Paclitaxel transport in HCT-8 and Caco-2 cells. Cells were grown on in Transwell dishes as described in Materials and Methods. [^{3}H]Paclitaxel, 0.1 μM (0.2 μCi/ml) was placed in the basolateral (B) or apical (A) chamber; media aliquots were taken at the indicated times and radioactivity was measured by liquid scintillation counting. Control Caco-2 (A) or HCT-8 (B) cells B to A (closed squares), control A to B (open squares), 5 μM CsA B to A (diamonds), 0.0025% TPGS B to A (triangles), 0.005% TPGS B to A (circles).

and colchicine, all established P-gp substrates. These data suggest that TPGS micelle formation in the intestinal lumen may not be the sole factor behind the increase in CsA absorption previously observed (20–22).

Several other surfactants, e.g. polysorbates, Cremophor EL, and Solutol 15, have been observed to be inhibitors of P-gp (38–41). These compounds are frequently added to pharmaceutical formulations to enhance solubility. These agents may also function to inhibit P-gp to add to their effect of enhancing drug absorption. Indeed the plasma concentrations of Cremophor EL in patients administered paclitaxel, which is formulated with this surfactant, reach levels sufficient to inhibit P-gp *in vitro* (42). The efficacy of this drug may, in part, be due to the activity of the Cremophor EL. Pluronic P85 has also recently been observed to block P-gp mediated rhodamine 123 efflux in Caco-2 and bovine brain microvessel endothelial cells (43). These data suggested that this agent may be useful for formulations to enhance brain and oral absorption.

TPGS has been used to enhance the bioavailability of CsA in liver transplant patients with the effects of significantly improving absorption and reducing daily drug cost. Sokol *et al.* and Boudreaux et al. reported increases in CsA absorption in pediatric transplant recipients treated with oral TPGS, 12.5 IU/kg and 10 IU/kg, respectively. The majority of patients receiving TPGS had previously experienced chronic cholestasis resulting in decreased bile flow suggesting poor solubilization of the lipophilic CsA. It was hypothesized that TPGS functioned as a bile substitute and solubilized the CsA through micelle formation, thus facilitating the absorption of the drug through the intestinal lumen. Similarly, Pan *et al.* reported a 28 and 32% decrease in CsA daily dose when co-administered with Liqui-E, a water soluble form of TPGS and a 26% decrease in daily CsA cost (44). Using normal healthy volunteers, Chang *et al.* observed a 60% rise in CsA area under the curve (AUC) in subjects receiving a TPGS-CsA cocktail. Decreased oral clearance and volume of distribution were also observed in those subjects. These authors proposed that the large, amphipathic TPGS may also be acting as an inhibitor of P-glycoprotein to enhance absorption and decrease transport back into the intestinal lumen. The current data support the hypothesis that one mechanism through which TPGS may enhance oral bioavailability is via inhibition of P-gp. Clearly further study on the effect of TPGS on oral drug delivery is required to confirm such a role.

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ADDRESS

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TITLE

ORAL FORMULATIONS OF CYTIDINE ANALOGS AND METHODS OF USE THEREOF

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Diagnosis is confirmed by presence of the Ph chromosome on cytogenic or molecular studies, although it is absent in 5% of patients.

During the accelerated phase of disease, anemia and thrombocytopenia usually develop. Basophils may increase, and granulocyte maturation may be defective. The proportion of immature cells and the leukocyte alkaline phosphatase score may increase. In the bone marrow, myelofibrosis may develop and sideroblasts may be seen on microscopy. Evolution of the neoplastic clone may be associated with development of new abnormal karyotypes, often an extra chromosome 8 or isochromosome 17.

Further evolution may lead to a blast crisis with myeloblasts (60% of patients), lymphoblasts (30%), and megakaryocytoblasts (10%). In 80% of these patients, additional chromosomal abnormalities occur frequently.

Prognosis

Before imatinib was used, with treatment 5 to 10% of patients died within 2 yr of diagnosis; 10 to 15% died each year thereafter. Median survival was 4 to 7 yr. Most (90%) deaths follow a blast crisis or an accelerated phase of the disease. Median survival after blast crisis is about 3 to 6 mo but can be up to 12 mo with remission.

Ph chromosome–negative CML and chronic myelomonocytic leukemia have a worse prognosis than Ph chromosome–positive CML. Their clinical behaviors resemble a myelodysplastic syndrome (see below).

Treatment

Except for some cases in which stem cell transplantation can be used successfully, treatment is not curative; however, survival can be prolonged by treatment with imatinib.

Imatinib inhibits the specific tyrosine kinase that results from the *ABL-BCR* gene product. It is dramatically effective in achieving complete clinical and cytogenetic remissions of Ph chromosome–positive CML and is clearly superior to other regimens (eg, interferon ± cytosine arabinoside). Imatinib also is superior to other treatments in the accelerated and blastic phases. Combinations of chemotherapy with imatinib in blast crisis have a higher response rate than does therapy with either approach alone. Treatment tolerance is excellent. The high level of durable complete remissions associated with imatinib therapy has led to the prospect of the cure of the disease.

Older chemotherapy regimens are reserved for *ABL-BCR*–negative patients, those who relapse after receiving imatinib, and those in blast crisis. The main agents are busulfan, hydroxyurea, and interferon. Hydroxyurea is easiest to manage and has the fewest adverse effects. The starting dosage is generally 500 to 1000 mg po bid. Blood counts should be followed q 1 to 2 wk and the dosage adjusted accordingly. Busulfan often causes unexpected general myelosuppression, and interferon causes a flu-like syndrome that often is unacceptable to patients. The main benefit of these therapies is reduction in distressing splenomegaly and adenopathy and control of the tumor burden to reduce the incidence of tumor lysis and gout. None of these therapies prolongs median survival > 1 yr compared with untreated patients; thus, reduction in symptoms is the major goal, and therapy is not continued in the face of significant toxic symptoms.

Although splenic radiation is rarely used, it may be helpful in refractory cases of CML or in terminal patients with marked splenomegaly. Total dosage usually ranges from 6 to 10 Gy delivered in fractions of 0.25 to 2 Gy/day. Treatment should begin with very low doses and careful evaluation of the WBC count. Response is usually disappointing.

Splenectomy may alleviate abdominal discomfort, lessen thrombocytopenia, and relieve transfusion requirements when splenomegaly cannot be controlled with chemotherapy or irradiation. Splenectomy has not proved to play a significant role during the chronic phase of CML.

MYELODYSPLASTIC SYNDROME

Myelodysplastic syndrome involves a group of disorders typified by peripheral cytopenia, dysplastic hematopoietic progenitors, a hypercellular bone marrow, and a high risk of conversion to AML. Symptoms are referable to the specific cell line most affected and may include fatigue, weakness, pallor (secondary to anemia), increased infections and fever (secondary to neutropenia), and increased bleeding and bruising (secondary to thrombocytopenia). Diagnosis is by blood count, peripheral blood smear, and bone marrow aspiration. Treatment with 5-azacytidine may help; if AML supervenes, it is treated per the usual protocols.

Etiology and Pathophysiology

Myelodysplastic syndrome (MDS) is a group of disorders, often termed preleukemia, refractory anemias, Philadelphia chromosome–negative chronic myelocytic leukemia, chronic myelomonocytic leukemia, or agnogenic myeloid metaplasia, resulting from a somatic mutation of hematopoietic precursors. Etiology is often unknown, but risk is increased with exposure to benzene, radiation, and chemotherapeutic agents (particularly long or intense regimens and those involving alkylating agents and epipodophyllotoxins).

MDS is characterized by clonal proliferation of hematopoietic cells, including erythroid, myeloid, and megakaryocytic forms. The bone marrow is normal or hypercellular, and ineffective hematopoiesis can cause anemia (most common), neutropenia, and/or thrombocytopenia. The disordered cell production is also associated with morphologic cellular abnormalities in bone marrow and blood. Extramedullary hematopoiesis may occur, leading to hepatomegaly and splenomegaly. Myelofibrosis is occasionally present at diagnosis or may develop during the course of MDS. Classification is by blood and bone marrow findings (see TABLE 142–4). The MDS clone is unstable and tends to progress to AML.

Symptoms and Signs

Symptoms tend to reflect the most affected cell line and may include pallor, weakness, and fatigue (anemia); fever and infections (neutropenia); and increased bruising, petechiae, epistaxis, and mucosal bleeding (thrombocytopenia). Splenomegaly and hepatomegaly are common. Symptoms may also be referable to other underlying diseases; eg, in an elderly patient with preexisting cardiovascular disease, anemia from MDS may exacerbate anginal pain.

Diagnosis

MDS is suspected in patients (especially the elderly) with refractory anemia, leukopenia, or thrombocytopenia. Cytopenias secondary to congenital disorders, vitamin deficiencies, or drug adverse effects must be ruled out. Diagnosis is by examining peripheral blood and bone marrow and identifying morphologic abnormalities in 10 to 20% of cells of a particular lineage.

Anemia is the most common feature, associated usually with macrocytosis and

TABLE 142–4. MYELODYSPLASTIC SYNDROME BONE MARROW FINDINGS AND SURVIVAL

CLASSIFICATION	CRITERIA	MEDIAN SURVIVAL (YR)
Refractory anemia	Anemia with reticulocytopenia, normal or hypercellular marrow with erythroid hyperplasia and dyserythropoiesis; blasts ≤ 5%	≥ 5
Refractory anemia with sideroblasts	Same as refractory anemia with ringed sideroblasts > 15% of NMC	≥ 5
Refractory anemia with excess blasts	Some cytopenia of ≥ 2 cell lines with morphologic abnormalities of blood cells; hypercellular marrow with dyserythropoiesis and dysgranulopoiesis; blasts = 5–20% of NMC	1.5
Chronic myelomonocytic leukemia	Same as refractory anemia with excess blasts with absolute monocytosis in blood; significant increase in marrow monocyte precursors	1.5
Refractory anemia with excess blasts in transformation	Refractory anemia with excess blasts and one or more of the following: ≥ 5% blasts in blood, 20–30% blasts in marrow, Auer rods in granulocyte precursors	0.5

NMC = nucleated marrow cells.

anisocytosis. With automatic cell counters, these changes are indicated by an increased MCV and RBC distribution width. Some degree of thrombocytopenia is usual; on blood smear, the platelets vary in size, and some appear hypogranular. The WBC count may be normal, increased, or decreased. Neutrophil cytoplasmic granularity is abnormal, with anisocytosis and variable numbers of granules. Eosinophils also may have abnormal granularity. Pseudo Pelger-Huët cells (hyposegmented neutrophils) may be seen. Monocytosis is characteristic of the chronic myelomonocytic leukemia subgroup, and immature myeloid cells may occur in the less well differentiated subgroups. The cytogenetic pattern is usually abnormal, with one or more clonal cytogenetic abnormalities often involving chromosomes 5 or 7.

Prognosis and Treatment

Prognosis depends greatly on classification and on any associated disease. Patients with refractory anemia or refractory anemia with sideroblasts are less likely to progress to the more aggressive forms and may die of unrelated causes.

Azacitidine improves symptoms, decreases the rate of transformation to leukemia and the need for transfusions, and probably improves survival. Other therapy is supportive, including RBC transfusions as indicated, platelet transfusions for bleeding, and antibiotic therapy for infection. In some patients, erythropoietin to support RBC needs, granulocyte colony-stimulating factor to manage severe symptomatic granulocytopenia, and, when available, thrombopoietin for severe thrombocytopenia can serve as important hematopoietic support but have not proved to increase survival. Allogeneic stem cell transplantation is useful, and nonablative allogeneic bone marrow transplantations are now being studied for patients > 50 yr. Response of MDS to AML chemotherapy is similar to that of AML, after age and karyotype are considered.

143 LYMPHOMAS

Lymphomas are a heterogeneous group of neoplasms arising in the reticuloendothelial and lymphatic systems. The major types are Hodgkin lymphoma and non-Hodgkin lymphoma (NHL)—see TABLE 143–1.

Lymphomas were once thought to be absolutely distinct from leukemias. However, better understanding of cell markers and keen tools with which to evaluate those markers now show that the differentiation between these 2 cancers is often vague. The notion that lymphoma is relatively restricted to the lymphatic system and leukemias to the bone marrow, at least in early stages, is also not always true.

HODGKIN LYMPHOMA

(Hodgkin's Disease)

Hodgkin lymphoma is a localized or disseminated malignant proliferation of cells of the lymphoreticular system, primarily involving lymph node tissue, spleen, liver, and bone marrow. Symptoms include painless lymphadenopathy, sometimes with fever, night sweats, unintentional weight loss, pruritus, splenomegaly, and hepatomegaly. Diagnosis is based on lymph node biopsy. Treatment is curative in about 75% of cases and consists of chemotherapy and/or radiation therapy.

In the US, about 7500 new cases of Hodgkin lymphoma are diagnosed annually. The male:female ratio is 1.4:1. Hodgkin lymphoma is rare before age 10 and is most common between ages 15 and 40.

Etiology and Pathophysiology

Hodgkin lymphoma results from the clonal transformation of cells of B-cell origin, giving rise to pathognomic binucleated Reed-Sternberg cells. The cause is unknown, but genetic susceptibility and environmental associations (eg, occupation, such as woodworking; history of treatment with phenytoin, radiation therapy, or chemotherapy; infection with Epstein-Barr virus, *Mycobacterium tuberculosis*, herpesvirus type 6, HIV) play a role. Risk is slightly increased in people with certain

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	C01	APARICIO et al., Current Opinion in Investigational Drugs, 2002, 3(4), 627–33.	
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LIST OF REFERENCES CITED BY APPLICANT (Use several sheets if necessary)		
	Application Number	12/466,213
	Filing Date	May 14, 2009
	First Named Inventor	Etter et al.
	Art Unit	1623
	Examiner Name	To be determined
	Attorney Docket No.	9516-847-999

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	C03	BEISLER et al., Journal of Medicinal Chemistry, 1977, 20(6), 806–12.	
	C04	BELLET et al., Cancer Chemotherapy Reports, Part I, 1974, 58(2), 217–22.	
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