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

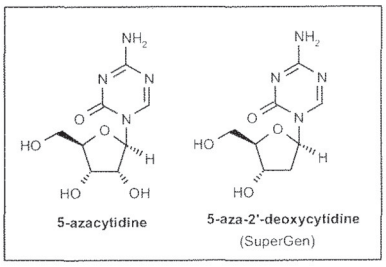
Methylation occurs after DNA replication by the addition of a methyl group from S-adenosylmethionine (SAM) to the 5'-position of cytosine residues. Approximately 3 to 4% of cytosines in mammalian DNA are methylated. Most of the 5-methylcytosine residues in eukaryotic DNA are found in the dinucleotide 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'-deoxcytidine, 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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