

Functional Diversity of DNA Methyltransferase Inhibitors in Human Cancer Cell Lines

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Abstract

DNA methyltransferase inhibitors represent promising new drugs for cancer therapies. The first of these compounds (5-azacytidine, Vidaza) has recently been approved as an antitumor agent, and others are presently in various stages of their preclinical or clinical development. Most of the archetypal inhibitors have been established and characterized in different experimental systems, which has thus far precluded their direct comparison. We have now established defined experimental conditions that allowed a comparative analysis of the six most widely known DNA methyltransferase inhibitors: 5-azacytidine (5-aza-CR), 5-aza-2'-deoxycytidine (5-aza-CdR), zebularine, procaine, (–)-epigallocatechin-3-gallate (EGCG), and RG108. Of these, 5-aza-CR, 5-aza-CdR, zebularine, and EGCG were found to exhibit significant cytotoxicity in human cancer cell lines. 5-aza-CdR and EGCG were also found to be genotoxic, as evidenced by the induction of micronuclei. In addition, 5-aza-CR, 5-aza-CdR, zebularine, and RG108 caused concentration-dependent demethylation of genomic DNA, whereas procaine and EGCG failed to induce significant effects. Finally, the experiments in cancer cell lines were complemented by a cell-free *in vitro* assay with purified recombinant DNA methyltransferase, which indicated that RG108 is the only drug capable of direct enzyme inhibition. These results show a substantial diversity in the molecular activities of DNA methyltransferase inhibitors and provide valuable insights into the developmental potential of individual drugs. (Cancer Res 2006; 66(5): 2794-800)

Introduction

DNA methylation plays an important role in the interpretation of genetic information (1). The question of whether a particular gene is methylated or not can therefore be of high relevance for cells, tissues, and organisms. Due to selective processes, tumor cells tend to hypermethylate and concomitantly silence tumor suppressor genes. Indeed, hypermethylated tumor suppressor genes represent one of the most consistent hallmarks of human cancers, and the phenomenon is of comparable significance to classic genetic mutations (2, 3). However, hypermethylation-associated epimutations need to be actively maintained after each cell division. This renders them a particularly attractive target for novel approaches in tumor therapy (4).

DNA methylation is catalyzed by a family of enzymes called DNA methyltransferases (5). The human genome contains four DNA

methyltransferase genes, *DNMT1*, *DNMT2*, *DNMT3A*, and *DNMT3B*, that encode proteins with distinct functional specificities. How these proteins become deregulated during cellular transformation has not been resolved yet. The process might involve both quantitative changes in DNA methyltransferase gene expression and aberrant enzyme targeting. Nevertheless, it has been established that the inhibition of DNA methyltransferase activity can strongly inhibit the formation of tumors. This is exemplified by the significantly reduced tumor burden of APC^{Min} with a heterozygous mutation of the *Dnmt1* gene (6). Similarly, DNA methyltransferase inhibitors have been shown to be effective in reverting the hypermethylation of tumor suppressor genes and suppressing cancer-specific cellular phenotypes (7).

The most widely used DNA methyltransferase inhibitor, 5-azacytidine (5-aza-CR), has been first characterized 25 years ago (8). This compound is a cytidine analogue that functions as a mechanism-dependent suicide inhibitor of DNA methyltransferases. To be effective, 5-aza-CR needs to be incorporated into DNA, which requires extensive modification of the compound through metabolic pathways. DNA methyltransferases recognize 5-azacytosine as natural substrate and initiate the methylation reaction. However, the analogue prevents the resolution of a covalent reaction intermediate and the enzyme thus becomes trapped and degraded (9). 5-aza-CR has proven to be effective in a phase III clinical trial (10) and has recently gained Food and Drug Administration approval for the treatment of myelodysplastic syndrome, a preleukemic bone marrow disorder.

5-aza-CR causes substantial cytotoxicity and is not a specific inhibitor of DNA methyltransferases. Due to its ribonucleoside structure, most of the compound becomes incorporated into RNA and thereby interferes with protein translation (11). This problem has been addressed by the development of a deoxyribonucleoside analogue, 5-aza-2'-deoxycytidine (5-aza-CdR; ref. 8), which becomes more directly incorporated into DNA and causes more efficient inhibition of DNA methyltransferases (12). 5-aza-CdR has also been tested in numerous clinical trials and has shown promising results in the treatment of myeloid leukemias (13–15). However, the substance is also characterized by significant cytotoxicity and low stability. These problems may be overcome by the use of zebularine, a novel nucleoside inhibitor with increased stability (16, 17). Zebularine has been reported to have comparatively little toxicity and seems to preferentially target tumor cells (17, 18).

Because nucleoside inhibitors may be inherently cytotoxic (19), much emphasis has been put on the development of compounds that target DNA methyltransferases more directly. Antisense-mediated or small interfering RNA-mediated degradation of *DNMT* mRNA represents one approach (20), although there are substantial procedural issues that remain unresolved. Another possibility is the development of small molecules that block DNA methyltransferase enzymes. For example, it has been suggested that

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the local anesthetic procaine inhibits DNA methyltransferases by perturbing interactions between the protein and its target sites (21). Furthermore, it has been proposed that the main polyphenol compound from green tea, (-)-epigallocatechin-3-gallate (EGCG), could block the catalytic pocket of the human DNMT1 enzyme (22). A similar mechanism of inhibition has also been suggested for RG108, the first rationally designed inhibitor of DNA methyltransferases (23).

In summary, there are currently six compounds (5-aza-CdR, 5-aza-CdR, zebularine, procaine, EGCG, and RG108) that have been characterized as DNA methyltransferase inhibitors. Particular inhibitory mechanisms have been proposed for each of these compounds, and they have all been shown to inhibit DNA methylation in human cancer cells. However, there has never been a comparative analysis of more than two inhibitors in uniform assay systems, which has thus far precluded a detailed assessment of drug properties. This study describes a comparative benchmark analysis of all six compounds to define their inhibitory properties and delineate their application potential.

Materials and Methods

Cell culture. TK6, Jurkat, and KG-1 cells were cultured in RPMI 1640 supplemented with 5% L-glutamine and 10% FCS (Invitrogen, San Diego, CA) or horse serum (TK6, Life Technologies, Gaithersburg, MD). HCT116 cells were cultured in McCoy's 5a medium supplemented with 10% FCS. Stock solutions (10-100 mmol/L) of 5-aza-CR (Sigma, St. Louis, MO), 5-aza-CdR (Calbiochem, La Jolla, CA), zebularine (Calbiochem), procaine (Sigma), EGCG (Sigma), and RG108 (23) were prepared by dissolving the respective substances in DMSO (zebularine), ethanol (RG108), or H₂O (all other compounds) and stored at -80°C. Stock solutions were diluted in cell culture media at the concentrations indicated.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Cellular proliferation was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously (24). Briefly, cells were incubated for 72 hours in inhibitor-supplemented medium, at the concentrations indicated; 25 μ L of MTT staining solution (2.5 mg/mL in PBS) were added to 1×10^4 cells in 100 μ L medium in 96-well culture plates and incubated for 4 hours at room temperature. After removal of the staining solution, 100 μ L of lysis solution (acetic acid/2-propanol, 1:20) was added, and lysates were incubated in the dark for another 1 hour at room temperature. Finally, absorbance was measured with a microtiter plate reader at 570 nm. In parallel experiments, cell numbers were directly determined by using a standard counting grid.

Cytotoxicity assays. For colony formation assays, HCT116 cells (500 per 100-mm dish) were plated in triplicate with inhibitor-containing medium at the concentrations indicated. Control plates were treated with 0.02% DMSO. After cell colonies became visible (13 days after plating), cells were fixed with 10% formaldehyde in PBS and stained with 0.1% crystal violet in PBS. Induction of apoptosis was analyzed by treating 1 to 2×10^5 TK6 cells/mL with various inhibitors, as indicated, followed by Annexin V and propidium iodide staining (25). For analysis, 0.5×10^6 cells were immersed in 100 μ L of binding buffer (Becton Dickinson, Sunnyvale, CA) that contained 0.5 μ g/mL Annexin V-Fluos and 20 μ g/mL propidium iodide. After 15 minutes of incubation at room temperature, 400 μ L of binding buffer were added before analysis. Samples were analyzed with a Becton Dickinson BD-LSR flow cytometer. The fluorescence of 2×10^4 cells was acquired and analyzed with CellQuest Pro software (Becton Dickinson). Cells with hypodiploid DNA content were identified by a cell sorting assay (26). Cells (1×10^5 TK6 or Jurkat, as indicated) were incubated for 72 hours in 3 mL medium under standard cell culture conditions. Cells were subsequently stained for 3 hours at 4°C in a buffer containing 50 μ g/mL propidium iodide, 0.1% tri-sodium citrate-2-hydrate, and 0.1% Triton X-100. Finally, 1×10^4 cells were sorted and analyzed using a Becton Dickinson FACSCalibur system.

Micronuclei formation assay. Induction of micronucleus formation was analyzed microscopically; 2×10^5 cells/mL (TK6 or HCT116, as indicated) were incubated in inhibitor-supplemented medium. Cell number was adjusted to 2×10^5 cells/mL after 24 and 48 hours with fresh, inhibitor-containing medium, to ensure exponential growth conditions, and cells were harvested after 72 hours. Control cells were mock-treated with 1% DMSO, the maximal concentration of solvent used for inhibitor treatment. For harvesting, cells were spun onto microscopic slides (Shandon Cytospin centrifuge), fixed in methanol (-20°C, 2 hours), and stained. For staining, slides were incubated with acridine orange [62.5 μ g/mL in Sorensen buffer: 67 mmol/L Na₂HPO₄/KH₂PO₄ (pH 6.8)] for 5 minutes, washed twice with Sorensen buffer for 5 minutes, and mounted for microscopy. One thousand cells were evaluated for the presence of micronuclei, which are round or oval shaped structures with no connection to the main nuclei, a size of <0.25, and similar staining characteristics as the main nuclei.

Determination of genomic DNA methylation levels. Genomic DNA was prepared from cells using the DNeasy kit (Qiagen, Chatsworth, CA). Global methylation levels were determined by capillary electrophoresis, as described previously (27). Briefly, genomic DNA was enzymatically hydrolyzed to single nucleotides, and the nucleotides were derivatized with the fluorescent marker BODIPY (Molecular Probes, Eugene, OR). Derivatized nucleotides were separated by capillary electrophoresis and analyzed in a Beckman P/ACE MDQ Molecular Characterization System.

Methylation analysis of the TIMP-3 promoter region. Genomic DNA was treated with sodium bisulfite (28) and analyzed by COBRA (29) using the primers TIMP-3_for AATCCCCAAACTCCAACCTAC and TIMP-3_rev TTTGTTTTTTTAGTTTTGTTTTT. PCR cycling conditions were 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 40 seconds for 35 cycles. The PCR product (301 bp) was digested with *Bst*UI (New England Biolabs, Beverly, MA). Digested PCR products were separated on agarose gels and quantified by densitometry. For bisulfite sequencing, PCR products were gel extracted and cloned using the TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions.

Reverse transcription-PCR. Total RNA was isolated from cells using Trizol reagent (Invitrogen), and cDNA was synthesized using the ThermoScript RT PCR system (Invitrogen); 20 μ L PCR reactions contained 2 μ L cDNA template, 1 \times ReddyMix buffer (Abgene, Rochester, NY), 10 μ mol/L each primer, 1 mmol/L deoxynucleotide triphosphates (Stratagene, La Jolla, CA), and 1.5 units of Thermoprime polymerase (Abgene). The primers used and the PCR conditions were TIMP-3 (forward, GCTGTGCAACTTCGTG-GAGAGG; reverse, CTCGGTACCAGTGCAGTAGCC) at 95°C for 3 minutes, 35 cycles (95°C for 30 seconds, 60°C for 30 seconds, 72°C for 45 seconds), and at 72°C for 5 minutes. Primers and PCR conditions for β -Amyloid have been described elsewhere (23).

In vitro DNA methyltransferase assay. To analyze the activity of test compounds in a cell-free *in vitro* system, a previously established DNA methyltransferase assay was used (23). A 798-bp fragment from the promoter region of the human *p16* gene was used as a template in a reaction mix containing M.SssI methyltransferase (New England Biolabs), S-adenosylmethionine, reaction buffer, and varying inhibitor concentrations. After completion of the methylation reaction, the DNA was column purified and digested for 3 hours at 60°C with the methylation-sensitive restriction enzyme *Bst*UI. The restriction reaction was analyzed on a 2% Tris-borate EDTA agarose gel.

Results

5-aza-CR, 5-aza-CdR, zebularine, procaine, EGCG, and RG108 represent six archetypal DNA methyltransferase inhibitors that have been shown to reverse epigenetic mutations in human cancer cells. Until now, a systematic analysis of these compounds has been precluded by their substantial chemical and pharmacologic heterogeneity. To directly compare DNMT inhibitors in human cancer cell lines, we had to establish drug concentrations that allowed a cross-inhibitor analysis in different cell lines. To this end, we used cell proliferation assays to determine the cellular IC₂₀

Table 1. Effects of DNMT inhibitors on the proliferation of various cancer cell lines

	TK6	Jurkat	KG-1	HCT116	Mean IC ₂₀
5-aza-CR	0.4	0.1	0.4	0.5	0.4
5-aza-CdR	0.1	0.05	0.1	0.3	0.1
zebularine	20	10	20	5	10
procaine	400	500	400	400	400
EGCG	10	20	20	10	10
RG108	20	40	20	20	20

NOTE: Numbers indicate cell line-specific IC₂₀ concentrations (in μmol/L) that caused a 20% reduction in cell proliferation after 3 days of growth in drug-supplemented medium.

concentration, which indicates the drug concentration that caused a 20% reduction in cell proliferation after 3 days of growth in inhibitor-supplemented media (Table 1). These particular experimental conditions were chosen because (a) 3 days of incubation are known to be sufficient for the reversion of epigenetic mutations by azanucleosides, and (b) cell viability is improved compared with more conventional IC₅₀ conditions. The proliferation experiments were done with a panel of four human cancer cell lines representing lymphoid (TK6 and Jurkat), myeloid (KG-1), or colorectal (HCT116) targets for epigenetic therapies. The results

revealed a strong variability in IC₂₀ concentrations between individual compounds (Table 1), ranging between 0.1 μmol/L (5-aza-CdR) and 400 μmol/L (procaine). However, IC₂₀ concentrations differed only slightly between individual cell lines and could therefore be used as drug-specific reference points for further experiments. For most compounds, IC₂₀ concentrations were in the concentration range that was previously reported to induce demethylation of epigenetically silenced genes. The only exception was zebularine, which was previously used at concentrations of 100 to 1,000 μmol/L (17, 18) and was now found to have an IC₂₀ concentration of 10 μmol/L. Of note, IC₂₀ concentrations also coincided with plasma concentrations that had been previously determined in pharmacokinetic studies (30–33), which suggests that they are similar to the concentrations that are to be used in epigenetic cancer therapies.

The observed effects of DNMT inhibitors on cell proliferation could be due to a slower cell cycle progression or to increased cytotoxicity. In an initial experiment to characterize the effect of individual inhibitors on cell growth, we used a colony formation assay (Fig. 1A). Equal numbers of HCT116 cells were plated on cell culture dishes, and the plating efficiency was determined after incubation with individual inhibitors at their respective IC₂₀ concentrations. This revealed a strong reduction in the plating efficiency for 5-aza-CdR and EGCG, a weaker reduction for CR and zebularine, and no significant reduction for procaine and RG108 (Fig. 1A). To characterize these effects in greater detail, we used three different assays that identified a variety of markers associated

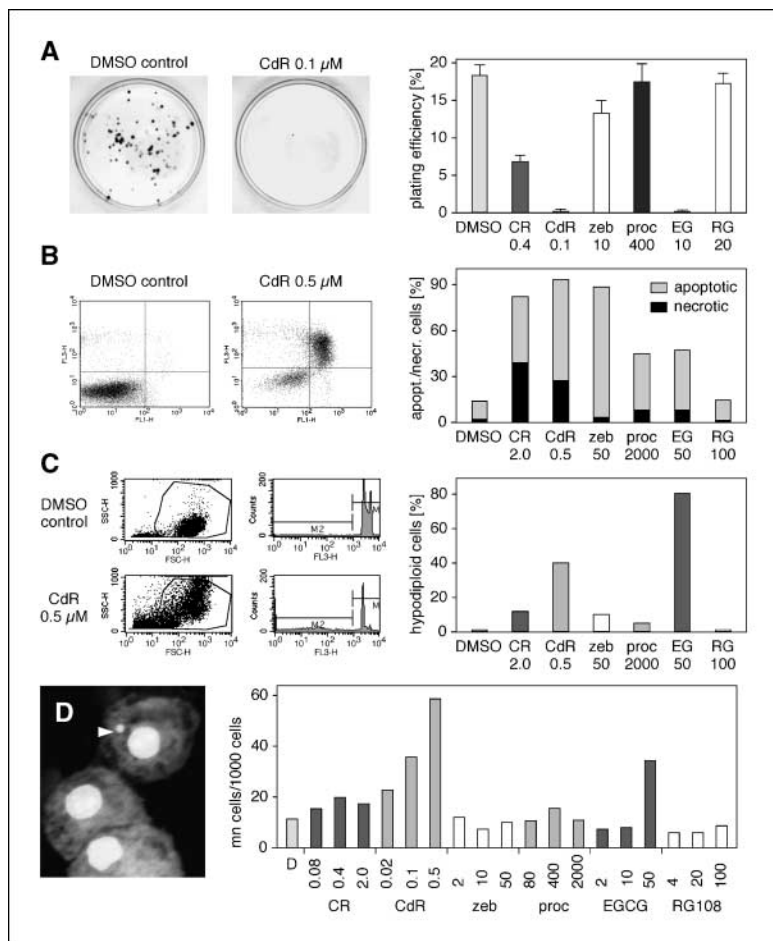
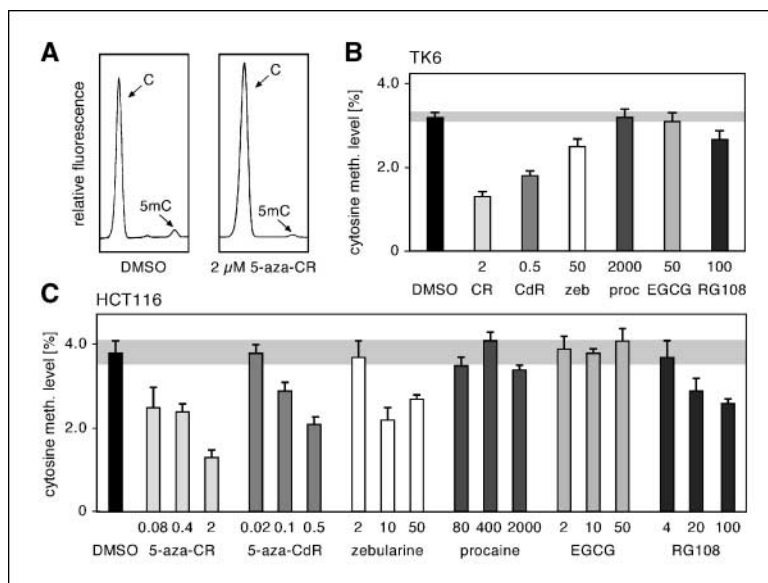


Figure 1. Characterization of cytotoxic effects induced by DNMT inhibitors. A, colony formation assay. HCT116 cells were plated in the presence of inhibitors or DMSO, and colonies were visualized by crystal violet staining. Inhibitor concentrations are indicated in μM. Columns, plating efficiency of multiple (>3) independent experiments; bars, SD. B, TK6 cells were grown in inhibitor-supplemented medium for 3 days, and apoptotic cells were identified by cell sorting after Annexin V (FL1-H) and propidium iodide (FL3-H) staining. Columns, fractionation of cells into apoptotic (Annexin V positive, gray) and necrotic (propidium iodide positive, black) populations. C, identification of cells with hypodiploid DNA content by cell sorting after propidium iodide staining. Columns, average frequency of hypodiploid cells, as determined in two independent experiments. D, identification of micronuclei (white arrowhead) by microscopic analysis of acridine orange-stained TK6 cells. Columns, average numbers of micronucleus-containing cells from two independent experiments.

Figure 2. Inhibition of cellular DNA methyltransferase activity by individual inhibitors. Human cancer cell lines were grown in inhibitor-supplemented medium. Genomic DNA was prepared after 3 days, and the cytosine methylation level was determined by capillary electrophoresis. **A**, representative electropherograms from HCT116 cells treated with DMSO (*left*, control) and 2 μM /L 5-azacytidine (*right*), respectively. **B**, effect of DNMT inhibitors at 5-fold IC_{20} concentrations on the genomic methylation level of TK6 cells. Inhibitor concentrations are indicated in μM . **C**, repeat experiment with HCT116 cells and varying inhibitor concentrations, centered around the drug-specific IC_{20} concentrations. *Bars*, SD; *horizontal gray column*, control methylation level (including SD).



with different aspects of cytotoxicity. These experiments were done with TK6 cells at different time points during treatment with various drug concentrations. The overall data showed a high level of consistency, and the most informative results were obtained after 3 days of drug incubation at 5-fold IC_{20} concentrations. In a first set of experiments, we determined for each compound its ability to induce apoptosis by using Annexin V and propidium iodide staining (Fig. 1B). Cell sorting revealed a clear (>2-fold) increase in the number of Annexin V-positive cells for all DNA methyltransferase inhibitors with the exception of RG108 (Fig. 1B). In a second set of experiments, we used a cell sorting assay that identified the proportion of cells with hypodiploid DNA content (Fig. 1C). This showed that the major fraction of cells treated with 5-aza-CdR or EGCG was found to be hypodiploid, whereas weaker effects were observed for 5-aza-CR, zebularine, and procaine but not for RG108 (Fig. 1C). Essentially, similar results were also obtained with Jurkat cells (data not shown). In a third set of experiments, we also investigated the drug-dependent formation of micronuclei (Fig. 1D), which represents a well-established end point for chromosomal damage in routine genotoxicity testing. Micronuclei from two independent experiments were scored microscopically after 3 days of inhibitor treatment (Fig. 1D). Under these conditions, we found a clear (>2-fold) induction of micronuclei for 5-aza-CdR (dose dependent) and EGCG (highest concentration, 50 μM /L), a weak effect with 5-aza-CR, and no significant evidence for micronuclei induction for zebularine, procaine, and RG108. Highly similar results were also obtained with HCT116 cells (data not shown). Our results thus indicate clear cytotoxic or genotoxic effects for all DNA methyltransferase inhibitors with the exception of RG108.

To further characterize the test compounds, we determined their effects on the genomic DNA methylation level. To this end, we incubated human cancer cell lines with drug-supplemented culture media for 3 days. Cells were then harvested, genomic DNA was isolated, and its chemically derivatized single nucleotides were analyzed by micellar electrokinetic capillary electrophoresis (Fig. 2A). Incubation of TK6 cells with 5-aza-CR or 5-aza-CdR at 5-fold IC_{20} concentrations revealed a methylation level decrease by 60% and 40%, respectively, when compared with controls (Fig. 2B).

A more moderate (20%) decrease was also observed with zebularine. EGCG or procaine did not cause a detectable effect on genomic DNA methylation levels, whereas RG108 resulted in a 20% decrease that was comparable with the effect induced by zebularine (Fig. 2B). To independently confirm these results in a second cell line, we analyzed each compound in HCT116 colon carcinoma cells and in three different concentrations centered around the respective drug-specific IC_{20} concentrations (Fig. 2C). The results revealed strong concentration-dependent demethylation for the nucleoside inhibitors 5-aza-CR, 5-aza-CdR, and zebularine. Interestingly, the drug-induced demethylation was stronger for 5-aza-CR than for 5-aza-CdR when both compounds were tested at equitoxic concentrations. Procaine and EGCG had no significant effect on the genomic methylation level, even at concentrations that were previously reported to be effective (21, 22). RG108 caused significant concentration-dependent demethylation that was in agreement with previously published results (23).

After having determined the effect of individual compounds on the whole-genome methylation, we also analyzed DNA methylation changes at a defined genomic locus. The promoter region of the *TIMP-3* tumor suppressor gene has been previously described to be completely methylated in HCT116 cells (34). This hypermethylation can be detected by combined bisulfite restriction analysis (COBRA), which is based on differential restriction enzyme cleavage of a PCR fragment amplified from bisulfite-deaminated genomic DNA. COBRA of genomic DNA from drug-treated HCT116 cells revealed significant concentration-dependent demethylation of the *TIMP-3* promoter after incubation with 5-aza-CR and 5-aza-CdR (Fig. 3A). For other inhibitors, the demethylation seemed to be weaker and potentially insignificant (Fig. 3A). Because demethylation of hypermethylated promoter regions is frequently accompanied by reactivation of gene expression, we analyzed the expression level of *TIMP-3* by semiquantitative reverse transcription-PCR. In agreement with the COBRA data, the results revealed detectable and concentration-dependent reactivation by 5-aza-CR and 5-aza-CdR (Fig. 3B). Other inhibitors did not cause a significant reactivation of *TIMP-3* at any of the concentrations tested (Fig. 3B). However, it remained possible that the local demethylation induced by zebularine, procaine, EGCG, or RG108

was too weak to allow the expression of *TIMP-3* and/or that the particular experimental conditions (3-day incubation of adherent cells in inhibitor-supplemented medium) favored the effects of azanucleosides. We therefore did bisulfite sequencing of multiple independent clones to determine the *TIMP-3* promoter methylation status more rigorously (Fig. 3C). In all clones sequenced, cytosines were restricted to CpG dinucleotides, which confirmed the stringent deamination of unmethylated cytosine residues during the bisulfite reaction. The sequencing results showed that the *TIMP-3* methylation level decreased from 92% to 78% after incubation with 2 $\mu\text{mol/L}$ 5-aza-CR, and to 56% with 0.5 $\mu\text{mol/L}$ 5-aza-CdR. No significant demethylation of the *TIMP-3* promoter could be observed with 50 $\mu\text{mol/L}$ zebularine, 100 $\mu\text{mol/L}$ RG108, 2000 $\mu\text{mol/L}$ procaine, or 50 $\mu\text{mol/L}$ EGCG. Together, these results provide additional evidence for a differential activity of the drugs tested in this study and indicate that 5-aza-CR and 5-aza-CdR might be most effective in reactivating tumor suppressor genes in cultured cell lines.

The diverse activities of individual DNMT inhibitors raised the possibility that the effects observed in cellular assays could be the consequence of drug-induced changes in cellular signaling. To characterize the inhibition of catalytic DNA methyltransferase activity in a more defined system, we used a cell-free *in vitro* assay. This assay visualizes the activity of the purified recombinant CpG methylase on a DNA template derived from the human *p16* promoter (23). The analysis of our test compounds over a broad range of inhibitor concentrations revealed no detectable effect for 5-aza-CR, 5-aza-CdR, and zebularine (Fig. 4). This is consistent with their requirement for incorporation into DNA that does not occur in a cell-free assay. EGCG was found to be strongly reactive and inactivated a variety of enzymes, including restriction endonucleases (data not shown). For this reason, EGCG could not be analyzed in our *in vitro* methylation assay. In agreement with our cellular assays, no effect was detectable for procaine (Fig. 4). In contrast, RG108 caused partial DNA methyltransferase inhibition at 40 and 200 nmol/L and complete inhibition at concentrations exceeding 200 nmol/L (Fig. 4). These results were in good

agreement with the previously reported IC_{50} concentration of 115 nmol/L (23). The ability to inhibit DNA methyltransferase activity in a cell-free *in vitro* assay distinguished RG108 from all other compounds analyzed in this study.

Discussion

DNA methyltransferase inhibitors represent promising new drugs for epigenetic cancer therapies (4, 7). Due to their longstanding availability, most of the attention has been focused on the cytosine analogues 5-aza-CR and 5-aza-CdR. These two drugs have been used in dozens of clinical trials and have been proven to be effective in a variety of hematologic disorders, especially when administered at low concentrations (10, 14, 15). More recently, other compounds have also been described to inhibit DNA methyltransferases, but their effectiveness has not been characterized in a systematic manner yet. In addition, the limited amount of available data has made it difficult to understand the particular characteristics of individual drugs. In this study, we have done a comparative benchmark analysis of compounds that have been previously reported to inhibit DNA methyltransferase activity in cancer cell lines (see Table 2 for a summary of results). This group of drugs did not include antisense oligonucleotides that function via DNA methyltransferase mRNA degradation (20, 35) or compounds that have been shown to inhibit DNA methyltransferases indirectly, either through their chemical reactivity (36) or through the inhibition of signaling pathways associated with DNA methylation (37).

Generally, the drugs analyzed in this study can be divided into two subgroups: nucleoside and non-nucleoside inhibitors (38). The first group consists of 5-aza-CR, 5-aza-CdR, and zebularine, three rather established drugs that function as suicide inhibitors after their incorporation into DNA. The second group contains procaine, EGCG, and RG108, three more experimental compounds that have been reported to inhibit DNA methyltransferases by interfering with enzyme activity. The effects of non-nucleoside inhibitors on cellular viability have not been analyzed systematically yet. Our results showed that EGCG induces pronounced cytotoxic effects.

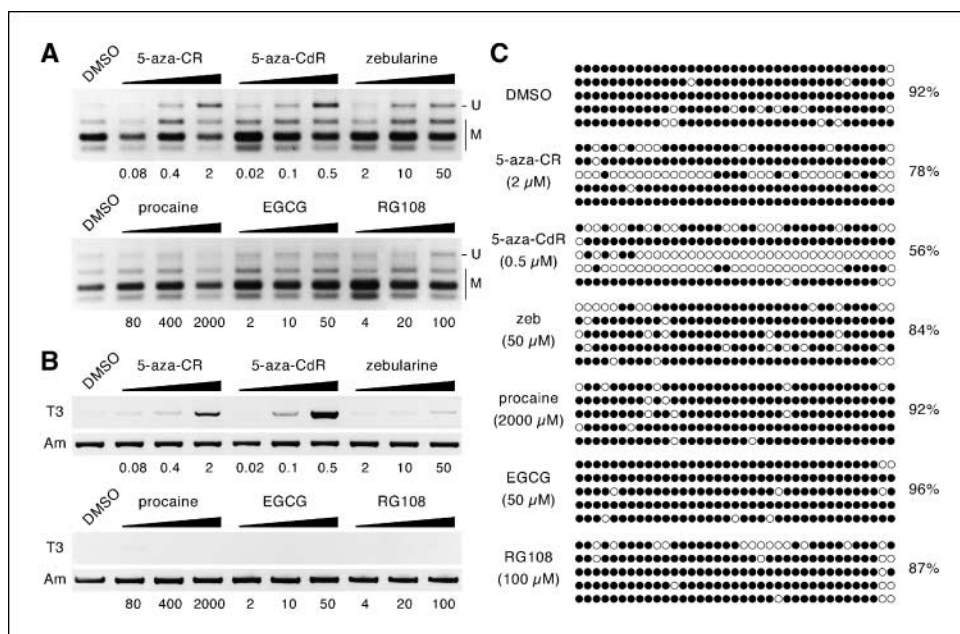


Figure 3. Demethylation and reactivation of *TIMP-3* by DNMT inhibitors. HCT116 cells were grown in inhibitor-supplemented medium. Genomic DNA was prepared after 3 days, and the methylation of the *TIMP-3* promoter region was determined. **A**, COBRA. Inhibitor concentrations (indicated in μM) are centered around the drug-specific IC_{20} concentrations, respectively. **B**, semiquantitative RT-PCR analysis of *TIMP-3* (*T3*) expression. β -Amyloid (*Am*) was used as a loading control. **C**, bisulfite sequencing analysis of the *TIMP-3* promoter region. Open circles, unmethylated CpG dinucleotides; closed circles, methylated CpG dinucleotides. Percentages indicate the fraction of methylated CpG dinucleotides.

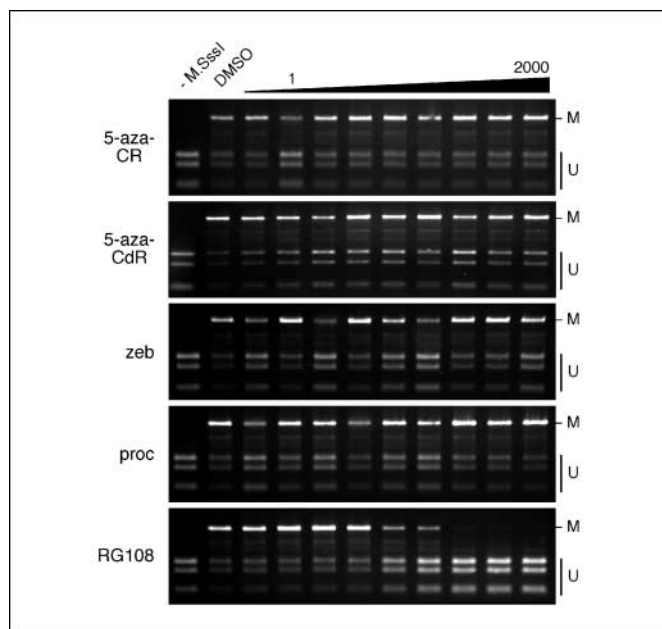


Figure 4. Inhibition of purified recombinant DNA methyltransferase. Incubation of an unmethylated DNA fragment with purified *SssI* methylase results in the generation of a methylated fragment (*M*) that is protected against restriction cleavage by *Bst*UI. Inhibition of DNMT activity generates unmethylated (*U*) fragments that are products of *Bst*UI restriction. Inhibitor concentrations are indicated (in *nM*, normalized to 1 nmol/L enzyme concentration).

However, EGCG did not inhibit DNA methylation, and the cellular effects can probably be attributed to the oxidative stress induced by this compound (39). Procaine showed intermediate proapoptotic activity at the highest drug concentrations (2,000 $\mu\text{mol/L}$) but was otherwise well tolerated by all cell lines tested in this study. Interestingly, RG108 showed no indications for cytotoxicity despite causing significant demethylation of genomic DNA, which indicates that demethylation, at least in the extent induced by RG108, is not intrinsically cytotoxic. These analyses were further expanded by the micronucleus induction assay, which provided a measure for drug genotoxicity. Although 5-aza-CR and some of its analogues were known to induce decondensation of heterochromatic regions and subsequent DNA strand breakage, leading to micronucleus formation (40, 41), zebularine and RG 108 did not

induce micronuclei under the conditions used in this study, despite effective hypomethylation. Possibly, other, non-heterochromatic DNA regions are preferably demethylated by these compounds (23), leading to less pronounced chromosomal instability. EGCG efficiently induced micronuclei without inhibiting DNA methylation, which can again be attributed to the strongly oxidizing properties of this compound. Of note, the micronucleus assay also suggested that the drug currently most favored for clinical applications (5-aza-CdR) had the highest genotoxicity. This indicates that epigenetic therapies could benefit from further optimization of treatment schedules and the clinical development of alternative drugs.

Despite their considerable cytotoxicity, azanucleoside inhibitors also showed the strongest demethylation effects, at least at concentrations exceeding the cellular IC_{20} value. In addition, 5-aza-CR and 5-aza-CdR were found to be the only drugs capable of significant demethylation and reactivation of the *TIMP-3* tumor suppressor gene, which is in agreement with the data provided by an independent study (42). The epigenetic changes induced by non-nucleoside inhibitors seemed to be more subtle. EGCG, for instance, did not show a detectable effect on cellular DNA methylation with any of the assays used in this study. This is notable because the compound has been reported to inhibit DNA methyltransferases in cell-free assays (22). However, EGCG also inactivated a variety of restriction enzymes in control assays, whereas none of these enzymes was affected by RG108 or other small molecules (data not shown). It is therefore possible that the *in vitro* activity of EGCG is due to its oxidizing ability and/or chemical reactivity (39). It has been shown that the degradation of EGCG under neutral to basic conditions (pH 6.8-7.8) generates strongly oxidizing hydroxyl radicals (43), which could influence the outcome of cell-free and cellular assays. Together, these results indicate that the effect of EGCG on DNA methylation might be more indirect than previously thought.

The data obtained with procaine also reveal novel details about the drug. It has been previously suggested that procaine might affect DNA methylation by perturbing the interaction between DNA methyltransferases and their CpG-rich target sequences (21). However, in this case, we should have observed an effect of the drug in our *in vitro* methylation assay that uses a CpG-rich substrate from the human *p16* promoter. We were also unable to detect a significant effect of procaine on DNA

Table 2. Effects of DNMT inhibitors in various assays

	Cytotoxicity*	Apoptosis induction [†]	Genotoxicity [‡]	Genomic demethylation	<i>TIMP-3</i> demethylation	<i>TIMP-3</i> reactivation	<i>SssI</i> inhibition
5-aza-CR	+	+	+	+	+	+	—
5-aza-CdR	+	+	+	+	+	+	—
zebularine	+	+	—	+	—	—	—
procaine	—	+	—	—	—	—	—
EGCG	+	+	+	—	—	—	NA
RG108	—	—	—	+	—	—	+

Abbreviation: NA, not applicable.

*Cytotoxicity was determined by colony formation assays.

[†]Apoptosis induction was determined by Annexin V staining.

[‡]Genotoxicity was determined by micronuclei formation assays.

methylation in TK6 and HCT116 cells, which is consistent with results published by others using primary mouse embryonic fibroblasts (44). It is possible that procaine affects DNA methylation only in a subset of human cell lines, like the MCF-7 breast cancer cell line (21).

Lastly, our combined results indicate the requirement for further development of zebularine and RG108. Both compounds caused a significant concentration-dependent demethylation of genomic DNA with comparatively little cytotoxicity. However, when analyzed more locally at the *TIMP-3* promoter, both compounds failed to induce significant demethylation and reactivation. This suggests that *TIMP-3* is not a primary target for zebularine-mediated or RG108-mediated demethylation. It should be pointed out that RG108-dependent demethylation and reactivation of *TIMP-3* has been observed after more prolonged drug incubation (23). It might be also possible to potentiate the relatively weak

effects of RG108 or zebularine by increasing the drug concentration (17). However, high concentrations would exceed the cellular IC₂₀ value and would thus be associated with substantially stronger cytotoxic effects. Of note, RG108 was the only compound tested in this study that was able to directly inhibit purified recombinant DNA methyltransferase. This result combined with its easily optimizable chemical structure establishes RG108 as an excellent candidate for further drug development.

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