Research Article

Epigenetic Reactivation of Tumor Suppressor Genes by a Novel Small-Molecule Inhibitor of Human DNA Methyltransferases

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Abstract

DNA methylation regulates gene expression in normal and malignant cells. The possibility to reactivate epigenetically silenced genes has generated considerable interest in the development of DNA methyltransferase inhibitors. Here, we provide a detailed characterization of RG108, a novel small molecule that effectively blocked DNA methyltransferases *in vitro* and did not cause covalent enzyme trapping in human cell lines. Incubation of cells with low micromolar concentrations of the compound resulted in significant demethylation of genomic DNA without any detectable toxicity. Intriguingly, RG108 caused demethylation and reactivation of tumor suppressor genes, but it did not affect the methylation of centromeric satellite sequences. These results establish RG108 as a DNA methyltransferase inhibitor with fundamentally novel characteristics that will be particularly useful for the experimental modulation of epigenetic gene regulation. (Cancer Res 2005; 65(14): 6305-11)

Introduction

Epigenetic modifications, like DNA methylation, play a major role in the interpretation of genetic information (1). This is exemplified by the establishment and maintenance of defined DNA methylation patterns that regulate the expression of cell type–specific genes (2). There are numerous examples for the presence of altered DNA methylation patterns in several pathologic conditions, including cancer (3). The acquisition of cancer-specific methylation patterns plays an important role in cellular transformation.

Genomic DNA methylation patterns of cancer cells are characterized by two distinct features. (*a*) Repetitive sequences like centromeric satellites tend to be hypomethylated, which has been linked to chromosome abnormalities (4). This has been supported by results derived from transgenic mice, where strongly reduced levels of DNA methylation were shown to cause genome instability and concomitant tumorigenesis (5). (*b*) CpG islands in the promoter regions of various genes become hypermethylated and the corresponding genes become silenced. Hypermethylation of tumor suppressor genes has been observed in all kinds of cancers and is generally assumed to be functionally equivalent to genetic loss-of-function mutations (6).

DNA methylation patterns are established and maintained by a family of enzymes called DNA methyltransferases (7). There are four known human DNA methyltransferases, DNMT1, DNMT2, DNMT3A, and DNMT3B, that use a highly conserved catalytic mechanism to methylate cytosine residues in the context of genomic DNA. DNA methyltransferases engage in a variety of specific protein-protein interactions that presumably determine their functional specificity. For example, it has been shown that DNMT1 can be associated with the replication machinery, which implies a function in the maintenance of DNA methylation patterns (8), whereas DNMT3A can be associated with transcription factors, which would be consistent with a role in *de novo* methylation (9). The analysis of DNMT knockout cells suggested a considerable level of cooperativity between individual enzymes (10, 11). This established an additional level of complexity that needs to be addressed in future experiments.

The possibility to reverse epigenetic mutations has generated considerable interest in the development of DNA methyltransferase inhibitors (12–14). The prototypical inhibitor 5-azacytidine has shown promising response rates in myelodysplastic syndrome patients (15) and has recently been approved for therapeutic treatment. However, all of the established inhibitors are mechanism based and require incorporation into genomic DNA (16–18). This inhibitory mechanism has been tightly linked to the high cytotoxicity of the compounds (19). There are a few additional substances that have been shown to reduce DNA methylation in human cells, but they do not seem to be specific inhibitors of DNA methyltransferases (20–22).

We have previously established a three-dimensional model of the human DNMT1 catalytic domain (23). This model was used for an in silico screen of a small-molecule database, which resulted in the identification of 2-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-3-(1*H*-indol-3-yl)propanoic acid as a candidate DNA methyltransferase inhibitor.[5] We have now established a protocol for the efficient synthesis of this compound, which we renamed to RG108. Subsequent testing in various assay systems revealed that RG108 efficiently blocked DNA methylation, both in a cell-free *in vitro* system and in human cancer cell lines. Additional experiments also revealed a significant, RG108-dependent demethylation and reactivation of epigenetically silenced tumor suppressor genes. Thus, RG108 represents a promising lead compound for the development of a novel class of DNA methyltransferase inhibitors that are designed to block the active site of these enzymes.

Materials and Methods

Chemical synthesis of RG108. The compound was synthesized in two steps with an overall yield of 90%. The intermediate product, a phthalic

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[5] P. Siedlecki, submitted.

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acid derivative with a protected and an activated ester group, methyl 2-((succinimidooxy)carbonyl)benzoate (24), was obtained from 2-(methoxycarbonyl)benzoic acid and *N*-hydroxysuccinimide in 90% yield after crystallization and drying. The structure was confirmed by mass spectrometry (electrospray ionization) and ^1H and ^{13}C nuclear magnetic resonance (NMR) analyses. RG108 was obtained by the reaction of methyl 2-((succinimidooxy)carbonyl)benzoate with L-tryptophan under basic conditions: Na_2CO_3 in water/acetonitrile, acidification with 2 N HCl, extraction in ethyl acetate, and evaporation of the solvent with an excellent yield of 100%. The pure yellow powder was analyzed by mass spectrometry (electrospray ionization) and ^1H and ^{13}C NMR to confirm the structure of RG108. Stock solutions were prepared by dissolving the substance in DMSO. For the synthesis of RG119, tryptamine (dissolved in acetonitrile) was used instead of L-tryptophan.

High-performance liquid chromatography analysis. RG108 and 5-azacytidine (Sigma, Schnelldorf, Germany) were dissolved in PBS buffer and kept in an incubator under standard cell culture conditions (37°C, 5% CO_2). Samples were taken at various time points between 0 and 28 days and stored at −80°C. For high-performance liquid chromatography (HPLC) analysis of RG108, we used a Reprosil-Pur C18 AQ 5 μm column (250/4 mm) and a 20%/80% to 70%/30% acetonitrile/5 mmol/L tetra-*N*-*n*-butylammonium dihydrogen phosphate buffer (pH 7.4) gradient over 30 minutes at 1 mL/min and UV detection at 220 nm. For HPLC analysis of 5-azacytidine, we used a Lichrocart-RP18 Lichrosphere 100 column (250/4 mm) and a 100% water to 100% acetonitrile gradient over 35 minutes at 1 mL/min and UV detection at or 220 and 240 nm. Each sample was measured thrice and the peaks were integrated with ChemStation software (Hewlett Packard, Waldbronn, Germany). The half-life was determined by linear regression analysis using standard procedures.

***In vitro* methylation assay.** The substrate DNA for the *in vitro* methylation assay was a 798 bp fragment (−423/+375 relative to the initiation codon) from the promoter region of the human $p16^{Ink4a}$ gene. The methylation reaction contained 350 to 400 ng substrate DNA and 4 units of M.SssI methylase (0.5 μmol/L, New England Biolabs, Frankfurt, Germany) in a final volume of 50 μL. Inhibitors were added to final concentrations of 10, 100, 200, and 500 μmol/L, respectively. Reactions were done at 37°C for 2 hours. After completion, the reaction was inactivated at 65°C for 15 minutes and the DNA was purified using the QIAquick PCR Purification kit (Qiagen, Hilden, Germany). Three hundred nanograms of purified DNA was digested for 3 hours at 60°C with 30 units of *Bst*UI (New England Biolabs) and analyzed on 2% Tris-borate EDTA agarose gels.

Cell culture. NALM-6 (25) and HCT116 (26) cells were cultured under standard conditions in RPMI 1640 and McCoy's 5a medium, respectively. To analyze the effect of DNA methyltransferase inhibitors, cells were cultivated in media supplemented with 5-azacytidine, RG108, or RG119, as indicated. Unless stated otherwise, cells were harvested after 96 hours for further analysis. For the determination of cellular growth and viability, cells were stained with trypan blue and counted using a standard counting grid.

Trapping assay. Frozen cell pellets (10^6-10^8 cells) were thawed on ice and resuspended in 1 mL ice-cold 1× PBS. After centrifugation (<1,000 × *g*) at 4°C for 5 minutes, the supernatant was removed and discarded. The pellet was resuspended with 100 to 200 μL ice-cold lysis buffer (150 mmol/L NaCl, 5 mmol/L EDTA, 50 mmol/L Tris-HCl (pH 8.0), 2 mmol/L phenylmethylsulfonyl fluoride, and 1% Igepal) and incubated on ice for 40 minutes. The lysate was centrifuged at 4°C for 15 minutes with 14,000 rpm. Supernatants were frozen in liquid nitrogen and stored at −80°C. Equal amounts of protein were then separated on 8% SDS-polyacrylamide gels and analyzed by Western blotting using standard procedures. The primary antibodies used were anti-DNMT1 (New England Biolabs; 1:2,000), anti-DNMT3b (Abgent, Bioggio, Switzerland, 1:250), and antiactin (Abcam, Cambridge, United Kingdom, 1:5,000). Primary antibodies were visualized by ECL chemiluminescence (Perkin-Elmer) according to the manufacturer's protocol.

DNA methylation analysis. Genomic DNA was isolated from cells using the DNeasy kit (Qiagen, Rodgau-Juegesheim, Germany). Genomic cytosine methylation levels were determined by capillary electrophoresis as described previously (27). The methylation status of satellite sequences was analyzed by methylation-sensitive Southern blots as described previously (11). Methylation-specific PCR analysis was done as previously described (28–30). Twenty-microliter reactions contained 2 μL template, 1× ReddyMix buffer (Abgene, Hamburg, Germany), 10 μmol/L of each primer, 1 mmol/L deoxynucleotide triphosphates (dNTP; Stratagene, Amsterdam, The Netherlands), and 1 unit of Thermoprime polymerase (Abgene). The primers (M: methylation specific, U: specific for unmethylated DNA) and the amplification programs were as follows: p16 (M-for TTATTAGAGGGTGGGGCGGATCGC, M-rev GACCCCGAACCGCGACCGTAA-; U-for TTATTAGAGGGTGGGGTGGATTGT, U-rev CAACCCCAAACCACAACCATAA), 95°C 3 minutes, 35 cycles (95°C 30 seconds, 60/65°C 30 seconds, 72°C 30 seconds), 72°C 5 minutes; TIMP-3 (M-for CGTTGCGTTTTATTTCGTTTCGTC, M-rev TACGCGCCGCCGACG; U-for TTGTTGTGTTTTATTTTGTTTTGTT, U-rev ATTACCATACACACCACCAACA), 95°C 3 minutes, 35 cycles (95°C 45 seconds, 52°C 45 seconds, 72°C 45 seconds), 72°C 5 minutes; SFRP1 (M-for GGTAGTAGTTTGCGGTCGCGGAGTC, M-rev GCCCGATACCCATACCGACTCTACG; U-for ATTGGGTAGTAGTTTGTGGTTGTGGAGTT, U-rev TACACCCAATACCCATACCAACTCTACA), 95°C 3 minutes, 35 cycles (95°C 30 seconds, 62°C 30 seconds, 72°C 30 seconds), 72°C 5 minutes. For quantitative methylation analysis, genomic DNA was deaminated with sodium bisulfite (31) and subsequently amplified by PCR using the following primers and PCR conditions: TIMP3-for TTTGTTTTTTTAGTTTTTGTTTTTT, TIMP3-rev AATCCCCCAAACTCCAACTAC, 95°C 3 minutes, 38 cycles (95°C 30 seconds, 58°C 30 seconds, 72°C 30 seconds), 72°C 5 minutes. SAT2-for ATGGAATTTTTATGAAATTGAAAT, SAT2-rev CATTCCATTAAATAATTCCATTC, 35 cycles (95°C 30 seconds, 51°C 30 seconds, 72°C for 30 seconds) 72°C 5 minutes. PCR products were purified using the QIAquick Gel Extraction kit (Qiagen). For combined bisulfite restriction analysis of chromosome 1 satellite 2, PCR fragments were digested with *Hin*fI and separated by agarose gel electrophoresis. For bisulfite sequencing of the *TIMP-3* promoter region, PCR fragments were subcloned into the pCR4-TOPO plasmid vector (Invitrogen) and subjected to automated sequencing. The CpG dinucleotides analyzed are localized in the region from -246 bp to -89 bp relative to the initiation codon of *TIMP-3*.

Reverse transcription-PCR. Total RNA was isolated from cells using Trizol reagent (Invitrogen) and cDNA was synthesized using the ThermoScript reverse transcription-PCR (RT-PCR) system (Invitrogen). RT-PCR was done as described previously (11, 30). Twenty-microliter reactions contained 2 μL template, 1× ReddyMix buffer (Abgene), 10 μmol/L of each primer, 1 mmol/L dNTPs (Stratagene), and 1.5 units of Thermoprime polymerase (Abgene). The primers used and the PCR conditions were as follows: p16 (forward TGGAGCCTTCGGCTGACT, reverse CTATGCGGGCATGGTTACTG) 95°C 5 minutes, 35 cycles (95°C 1 minute, 58°C 1 minute, 72°C 1 minute), 72°C 5 minutes; TIMP-3 (forward GCTGTGCAACTTCGTGGAGAGG, reverse CTCGGTACCAGCTGCAGTAGCC) 95°C 3 minutes, 35 cycles (95°C 30 seconds, 60°C 30 seconds, 72°C 45 seconds), 72°C 5 minutes; SFRP1 (forward CCAGCGAGTACGACTACGTGAGCTT, reverse CTCAGATTTCAACTCGTTGTCACAGG) 95°C 5 minutes, 35 cycles (95°C 1 minute, 62°C 1 minute, 72°C 1 minute), 72°C 5 minutes; β-amyloid (forward GTGAAGATGGATGCAGAATTCCG, reverse AAAGAACTTGTAGGTTGGATTTTCG) 95°C 3 minutes, 35 cycles (95°C 45 seconds, 60°C 1 minute, 72°C 45 seconds), 72°C 5 minutes.

Molecular modeling of RG119. RG119 was identified by producing a series of compounds with various minor changes to the functional groups of RG108. Compounds were created with CORINA (32), assigned Gasteiger-Huckel charges, and minimized before docking. The corresponding structures were docked into the active site of our DNMT1 model (23) using previously established parameters for DOCK v5.1.0 and scored by minimization procedures.

Results

The identification and development of small molecules that block the active sites of human DNA methyltransferases represents a novel strategy in the design of epigenetic modifier compounds. The establishment and experimental validation of a three-dimensional model for the DNMT1 catalytic domain (23) provided

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us with an opportunity to screen virtual databases for candidate compounds that inhibit human DNA methyltransferases in a mechanism-independent manner. A screen against the prefiltered Diversity Set of the National Cancer Institute database suggested that phthalimidotryptophan-like molecules might be particularly effective in inhibiting DNA methyltransferase activity.[6] To further characterize the efficacy of these compounds, we synthesized RG108 (Fig. 1*A*), a molecule that was predicted to strongly interact with the DNMT1 active site. Synthesis was done in two steps from common educts with an overall yield of 90% (Fig. 1*B*) and several hundred milligrams of the compound were obtained in the form of pure yellow crystals. HPLC analysis showed that the half-life of RG108 was ~20 days in neutral aqueous solutions at 37°C (Fig. 1*C*). Under the same experimental conditions, the half-life of the prototypical DNA methyltransferase inhibitor, 5-azacytidine, was only 17 hours (Fig. 1*C*). This is in agreement with the previously reported instability of 5-azacytidine (33), which represents a major impediment to its practical application.

As an initial step toward the characterization of RG108, we analyzed the effect of the compound in a cell-free *in vitro* DNA methylation assay. For this assay, we used the purified recombinant CpG methylase M.SssI. This enzyme is distinguished by a robust activity and also shows significant structural similarities with the DNMT1 catalytic domain.[7] A 798 bp PCR fragment from the promoter region of the human *p16*^{Ink4a} gene was used as a substrate and DNA methylation was visualized by digestion with the methylation-sensitive restriction enzyme *Bst*UI. Thus, inhibition of DNA methyltransferases can be detected by the appearance of unprotected, smaller restriction fragments (Fig. 2*A*). A direct comparison of RG108 and 5-azacytidine in this assay revealed a readily detectable inhibitory effect for RG108, but not for 5-azacytidine (Fig. 2*A*). This result is consistent with the assumption that RG108 is able to inhibit the free DNA methyltransferase, whereas 5-azacytidine needs to be incorporated into DNA (17, 34). Additional experiments showed that increasing concentrations of RG108 resulted in increasing amounts of unprotected *Bst*UI restriction fragments (Fig. 2*B*). To our knowledge, this represents the first example for the pharmacologic inhibition of a purified DNA methyltransferase *in vitro*. Densitometric quantification of fragment intensities confirmed a significant, concentration-dependent inhibition by RG108. After a normalization to 1 nmol/L enzyme concentration, these data could also be used to determine the IC_{50} value of RG108 at 115 nmol/L (Fig. 2*C*).

Mechanism-based inhibitors, like 5-azacytidine, have been shown to covalently trap DNA methyltransferases, which has been linked to the significant toxicity of these compounds. To characterize the effect of RG108 on the viability of human cell lines, we incubated HCT116 and NALM-6 cells with our inhibitor. We supplemented tissue culture media with 10 μmol/L RG108 and incubated cells over 15 days. Media without inhibitor and media supplemented with 10 μmol/L 5-azacytidine were used for controls. The results showed that RG108 had no effect on the viability of either cell line (Fig. 3*A*). In contrast, 5-azacytidine showed an intermediate effect on HCT116 cells and seemed to be highly toxic to NALM-6 cells (Fig. 3*A*). We then sought to analyze the effect of RG108 on genomic DNA methylation. To this end, genomic DNA was isolated from HCT116 and NALM-6 cells and the cytosine methylation level was determined by capillary electrophoresis. We also obtained DNA from parallel experiments with 5-azacytidine. Due to the high toxicity of 5-azacytidine, reliable DNA methylation values could only be obtained for HCT116 cells (after 5 days of incubation) and showed a 50% reduction. Analysis of genomic DNA from RG108-treated cells revealed a significant demethylation of HCT116 cells after 5 days (20%) and 15 days (30%). Demethylation of genomic DNA was also observed in NALM-6 cells, where incubation with RG108 reduced DNA methylation by 10% (after 5 days) and 30% (after 15 days), respectively (Fig. 3*B*). To further characterize the inhibitory mechanism of RG108, we analyzed trapping of DNA methyltransferases by visualizing the depletion of the enzymes from cell extracts (35, 36). HCT116 cells were incubated with RG108 or an equal concentration of 5-azacytidine and protein extracts were analyzed for the presence of the major DNA methyltransferases (11), DNMT1 and DNMT3B, and actin

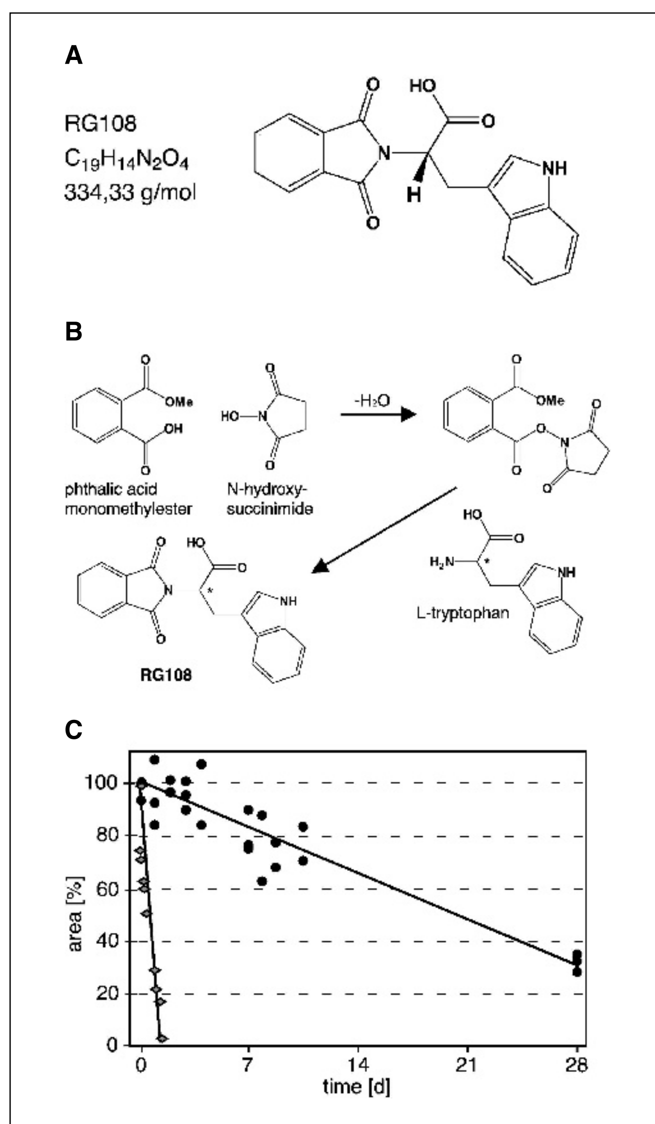

Figure 1. Structure and stability of RG108. *A,* structure of RG108. *B,* synthesis of RG108. *C,* degradation of RG108 (●) and 5-azacytidine (◇) in neutral phosphate buffer at 37°C. Samples were taken at various time points and analyzed by HPLC. The quantification of peak areas indicated a linear degradation of both compounds. The mean half-life was calculated to be 20 days for RG108 and 17 hours for 5-azacytidine.

[6] P. Siedlecki, submitted.
[7] P. Siedlecki, unpublished data.

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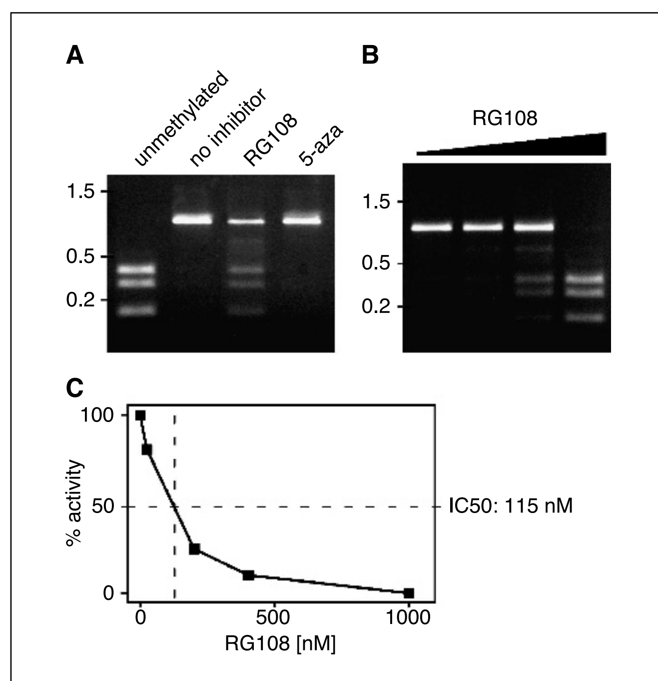

Figure 2. Inhibition of purified recombinant DNA methyltransferase activity by RG108. *A, in vitro* methylation assay. Equal amounts of purified SssI methylase were incubated with an unmethylated DNA fragment. The addition of RG108 resulted in a detectable decrease in DNA methyltransferase activity, as visualized by the appearance of small *Bst*UI restriction fragments. No inhibitory effect was observed with equal concentrations of 5-azacytidine. *B,* increasing amounts of RG108 progressively inhibit methyltransferase activity. *C,* determination of enzyme activity by densitometric quantification of restriction fragments and after normalization of enzyme concentrations to 1 nmol/L. The results indicate an IC_{50} value of 115 nmol/L.

(as a control). This showed that DNA methyltransferase levels were not affected by RG108 (Fig. 3*C*), which suggested that RG108 does not result in covalent trapping of DNA methyltransferases. In contrast, 5-azacytidine resulted in a strong depletion of DNMT1 and potentially also in a weaker reduction in DNMT3B levels (Fig. 3*C*). These results are consistent with the direct inhibition observed *in vitro* and provide a major distinction between RG108 and standard mechanism-based inhibitors of DNA methyltransferases.

To confirm the specificity of the DNA methyltransferase inhibition by RG108, we also looked for derivatives with a potentially reduced inhibitory activity. Molecular modeling suggested a critical role of the carboxyl group of RG108 in its interaction with the DNMT1 active site (Fig. 4*A*). Docking calculations also revealed a strong dependency of the binding energy on the presence of the carboxyl group (Fig. 4*B*). Therefore, we synthesized RG119, a derivative that lacks the central carboxyl group, but is otherwise identical to RG108 (Fig. 4*C*). When tested in our cell culture assay with HCT116 cells, RG119 failed to demethylate genomic DNA (Fig. 4*D*). These results establish an important role for the carboxyl group of RG108 in the interaction with the active site of the enzyme and suggest a considerable specificity in the interaction between the inhibitor and the DNA methyltransferase active site.

To analyze the effect of RG108 on the proliferation of HCT116 cells, we varied the inhibitor concentration from 1 to 100 μmol/L and determined the proliferation rate by cell counting. Whereas untreated HCT116 cells doubled five times in 5 days, 100 μmol/L RG108 decreased the cell division rate by 30% to 3.6 doublings (Fig. 5*A*). Staining of cells with trypan blue failed to detect a significant number of dead cells, even at 100 μmol/L RG108 (data not shown), which was again consistent with a low toxicity of the compound. Reduced proliferation of 5-aza-2′-deoxycytidine–treated cells has previously been linked to the epigenetic reactivation of the $p16^{Ink4a}$ tumor suppressor gene (37). HCT116 cells contain both a hypermethylated wild-type allele and an unmethylated mutant allele (28). When we sequenced cloned RT-PCR products from the *p16* locus, wild-type transcripts were only detectable in RG108-treated cells, but not in control cells (Fig. 5*B*), which strongly suggested an epigenetic reactivation of the *p16* gene. Together, these results provided the first indication for a connection between RG108-mediated demethylation and the reactivation of epigenetically silenced tumor suppressor genes. To characterize the epigenetic changes of RG108-treated cells in greater detail, we did methylation-specific PCR for three cancer-related genes that are epigenetically silenced in HCT116 cells: the $p16^{Ink4a}$ tumor suppressor gene, the gene encoding secreted frizzled-related protein 1 (*SFRP1*; ref. 30), and the putative tumor suppressor gene *TIMP-3* (29). For all three loci, we observed a detectable demethylation upon incubation with RG108 (Fig. 5*C*). We then used RT-PCR to analyze the effect of RG108 on the expression of all three genes and observed a significant RG108-dependent reactivation in all cases (Fig. 5*C*). Lastly, RG108-dependent demethylation of a hypermethylated tumor suppressor gene was also confirmed quantitatively by bisulfite sequencing of the *TIMP-3* promoter region. This revealed a significantly higher number of unmethylated CpG dinucleotides in RG108-treated

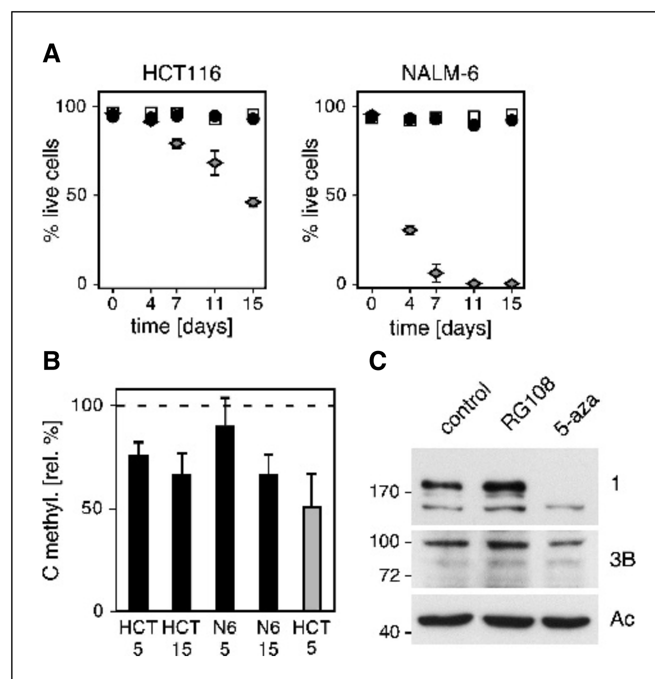

Figure 3. The effect of RG108 on human cancer cell lines. *A,* viability of HCT116 (*left*) and NALM-6 cells (*right*) in medium supplemented with 10 μmol/L RG 108 (●) or 10 μmol/L 5-azacytidine (◆). □, controls. *Bars,* SD. *B,* determination of genomic cytosine methylation levels. Methylation levels of NALM-6 (*N6*) and HCT116 (*HCT*) cells incubated with 10 μmol/L RG108 (*black columns*) for 5 and 15 days, respectively, are indicated relative to untreated control cells. *Gray column,* results from a parallel experiment with 10 μmol/L 5-azacytidine. *C,* trapping assay. HCT116 cells were incubated with equal concentrations of RG108, 5-azacytidine, or without inhibitor. Protein extracts were then probed for the presence of DNMT1 (*1*), DNMT3B (*3B*), and actin (*Ac*) by Western blotting. This revealed the covalent trapping of DNA methyltransferase proteins by 5-azacytidine, but not by RG108. The lower band in DNMT1 blots is presumably derived from an unrelated protein.

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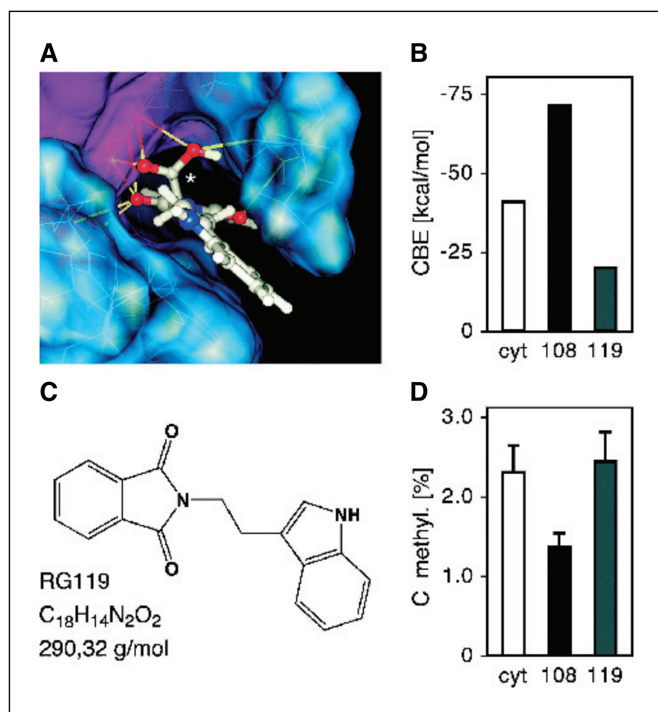

Figure 4. Structure-function relationships of RG108. *A,* docking of RG108 into the catalytic pocket of DNMT1 suggests an important role of the carboxyl group (*) in the interaction between enzyme and inhibitor. *Yellow lines,* predicted hydrogen bonds. *B,* calculated binding energies (*CBE*) of cytidine (*white column*), RG108 (*black column*), and RG119 (*gray column*), a derivative of RG108 that lacks the carboxyl group, docked into the DNMT1 active site. *C,* structure of RG119. *D,* genomic cytosine methylation levels of HCT116 cells incubated with 10 μmol/L RG119 (*gray column*) are compared with methylation levels of corresponding cells incubated without inhibitor (*white column*), or 10 μmol/L RG108 (*black column*). *Bars,* SD.

HCT116 cells (Fig. 5*D*). Together, these results show that RG108 is able to revert epigenetic mutations in HCT116 cells.

It has been speculated that chromosomal instability could be promoted by drug-induced demethylation of genomic DNA (5). In addition, it has been suggested that demethylation of centromeric satellite elements might play an important role in chromosomal instability (4). Therefore, we analyzed the methylation status of chromosome 18 α satellite and chromosome 1 satellite 2 sequences after incubation of HCT116 cells with RG108 or 5-azacytidine. Methylation-sensitive genomic Southern blots revealed substantial demethylation of both repetitive sequences after incubation with 10 μmol/L 5-azacytidine, whereas 10, 30, or 100 μmol/L RG108 failed to cause a detectable effect (Fig. 6*A*). The differential sensitivity of chromosome 1 satellite 2 elements toward RG108 and 5-azacytidine was also confirmed by combined bisulfite restriction analysis (Fig. 6*B*). No methylation changes were detectable after treatment with RG108, whereas parallel incubation with 5-azacytidine caused significant demethylation (Fig. 6*B*). In summary, our results thus indicate that RG108 might have a more specific demethylating activity than 5-azacytidine.

Discussion

The reversion of epigenetic mutations by DNA methyltransferase inhibitors represents an experimental strategy with great promise for cancer therapy (13). Until now, this approach has been mainly based on the use of 5-azacytidine and its derivatives. However, most of these inhibitors are characterized by a significant toxicity, which has restricted their clinical application (38, 39). These problems have been addressed by the development of zebularine, a chemically stable cytidine analogue (40). Whereas zebularine seems to be less toxic than azacytosine nucleotides, it also utilizes a covalent trapping mechanism for the inhibition of DNA methyltransferases (18, 36). Due to the inherent cytotoxicity of this mechanism (41), it was important to identify chemical compounds with an ability to directly block human DNA methyltransferases. We have now characterized RG108, the first DNA methyltransferase inhibitor produced by rational drug design. The further development of this compound will provide novel opportunities for the reactivation of epigenetically silenced genes.

Recently, several groups have reported demethylating activities for a few other small molecules. For example, it was shown that the antiarrhythmic drug procainamide and the local anesthetic procaine induce demethylation at high micromolar concentrations (20, 42). The inhibitory mechanism is still unclear, but it

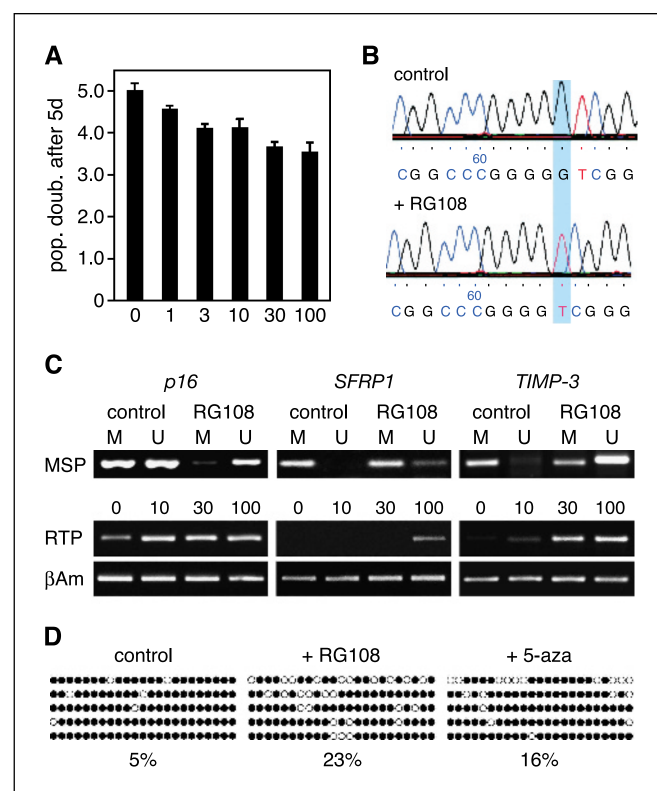

Figure 5. RG108 demethylates and reactivates tumor suppressor genes. *A,* RG108 inhibits the proliferation of HCT116 cells. Equal amounts of cells were incubated with different concentrations of RG108 and counted after 5 days. *Columns,* results from three independent experiments; *bars,* SD. *B,* reactivation of the *p16[Ink4a]* gene in HCT116 cells treated with RG108. *Top,* sequence of a wild-type RT-PCR product from cells treated with 10 μmol/L RG108. *Bottom,* untreated HCT116 cells expressed only the mutant allele of *p16. C,* effect of RG108 on the methylation and expression of various epigenetically silenced genes in HCT116 cells. Methylation-specific PCR (*MSP*) was used to analyze the methylation status of *p16[Ink4a], SFRP1*, and *TIMP-3* in DNA from cells incubated with 10 μmol/L RG108. RT-PCR (*RTP*) was used to determine the expression level in cells incubated with 0, 10, 30, or 100 μmol/L RG108 as indicated. β-amyloid (*βAm*) was used as a loading control. *D,* bisulfite sequencing analysis of the *TIMP-3* promoter region in HCT116 cells treated with 10 μmol/L RG108 or 10 μmol/L 5-azacytidine, respectively. ●, methylated cytosine residues; ○, unmethylated cytosine residues; numbers indicate the percentage of unmethylated cytosine residues relative to the total number of cytosines. The demethylation effect caused by RG108 was significant ($P < 0.01$) as determined by a *t* test.

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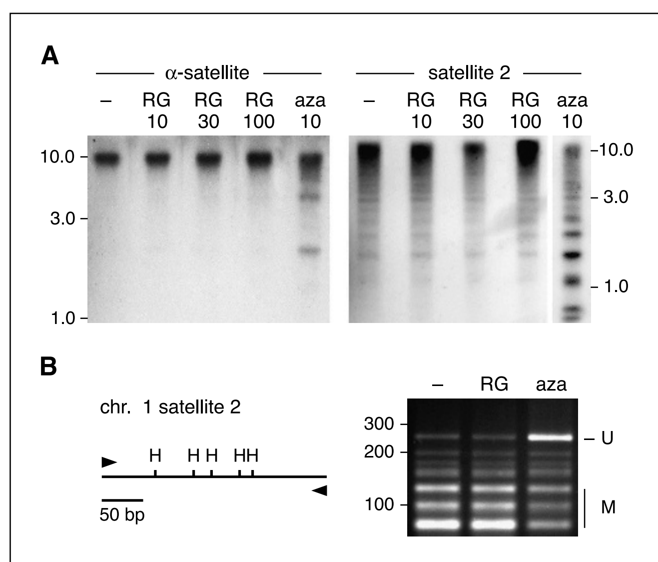

Figure 6. Differential sensitivity of centromeric satellite elements toward RG108 and 5-azacytidine. *A,* methylation-sensitive Southern analysis of centromeric satellite elements. HCT116 cells were incubated with variable concentrations of RG108 (*RG*) or 5-azacytidine (*aza*), respectively. The size of marker fragments is indicated (in kbp). *B,* combined bisulfite restriction analysis of chromosome 1 satellite 2 elements. *Arrowheads,* position of PCR primers; *H,Hin*fI restriction sites that were used for methylation analysis.- DNA was analyzed from HCT116 cells treated with 10 μmol/L RG108 or 10 μmol/L 5-azacytidine. The "U" band represents the completely unmethylated epiallele. "M" bands represent (partially) methylated epialleles. The size of marker fragments is indicated (in bp).

might involve the binding of the substances to CG-rich DNA sequences (20). In addition, two complex organic compounds, (−)-epigallocatechin-3-gallate (21) and psammaplins (22), were also reported to have demethylating activity. However, no evidence for a direct inhibition of methyltransferases was provided for either substance and it is possible that their pharmacologic effect on DNA methylation is rather indirect. In this context, it is also worth mentioning that none of these substances seems to be structurally related to RG108.

RG108 represents a previously uncharacterized compound that was selected for its ability to block the active site of the human DNMT1 enzyme in an in silico model. The results from our cell-free *in vitro* assay using purified recombinant CpG methylase are also consistent with such an inhibitory mechanism. The strong dependence of the methylation inhibition on the carboxyl group of RG108 may suggest a considerable specificity of the compound for DNA methyltransferases. Because RG108 was isolated through a screen with a three-dimensional model of DNMT1, it is possible that this enzyme represents the preferential target. However, the catalytic domains of the four human DNMT enzymes are highly conserved, which would predict similar interactions with RG108. The precise specificity of the inhibitor remains to be analyzed in future experiments and might be further refined by the design of optimized derivatives.

RG108 was able to reactivate several epigenetically silenced tumor suppressor genes in a human colon cancer cell line. At the same time, the compound did not seem to alter the methylation status of centromeric repeats. This might provide a considerable advantage for the maintenance of chromosome stability in demethylated cells, which has been shown to be affected by the hypomethylation of satellite DNA (4, 5). The potential preference of RG108 for euchromatic regions might suggest that euchromatic and heterochromatic sequences are methylated by distinct pools of DNA methyltransferases. Whether this involves differences in the functional specificities of individual DNA methyltransferases (43), differences in their catalytic properties (44, 45) or differences in their regulation during the cell cycle (46) remains to be determined. The latter possibility is consistent with the recent observation of replication-independent loading of DNMT1 onto centromeric heterochromatin (47). Centromeric heterochromatin and euchromatic genes may thus be methylated by separate pathways with different sensitivities toward RG108.

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