Down-regulation of Human DNA-(Cytosine-5) Methyltransferase Induces Cell Cycle Regulators p16\textsuperscript{ink4A} and p21\textsuperscript{WAF/Cip1} by Distinct Mechanisms*

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A common event in the development of human neoplasia is the loss of growth regulatory tumor suppressor functions. Methylation of 5′ CpG islands of tumor suppressor genes and elevated levels of the DNA-(cytosine-5)-methyltransferase enzyme (DNA MeTase) are also prevalent features of human neoplasia. However, direct evidence that elevated DNA MeTase levels alter gene expression and influence oncogenesis has been difficult to obtain, in part due to the lack of specific DNA MeTase inhibitors. Here we show that specific reduction of cellular DNA MeTase levels in human cancer cells with potent antisense inhibitors: 1) causes demethylation of the p16\textsuperscript{ink4A} gene promoter; 2) causes re-expression of the p16\textsuperscript{ink4A} protein; 3) leads to accumulation of the hypophosphorylated form of the retinoblastoma protein (pRb); and 4) inhibits cell proliferation. Stepwise reduction of cellular DNA MeTase protein levels also induced a corresponding rapid increase in the cell cycle regulator p21\textsuperscript{WAF/Cip1} protein demonstrating a regulatory link between DNA MeTase and the growth regulator p21\textsuperscript{WAF/Cip1} that is independent of methylation of DNA. These results suggest that the elevated levels of DNA MeTase seen in cancer cells can inhibit tumor suppressors by distinct mechanisms involving either transcriptional inactivation through DNA methylation or by a methylation independent regulation.

In mammals, methylation of the 5′ position of cytosine in the CpG dinucleotide sequence is the only naturally occurring covalent modification of the genome. The enzyme DNA 5′-cytosine methyltransferase (DNA MeTase)\textsuperscript{1} catalyzes the transfer of a methyl group from \textit{S}-adenosylmethionine to the 5′ position of cytosines residing in the dinucleotide sequence CpG (1). DNA methylation patterns correlate inversely with gene expression (2) and, therefore, DNA methylation has been suggested to be an epigenetic determinant of gene expression. However, experimental inhibition of the DNA MeTase has relied for the most part on the nucleoside analogs 5-azacytidine (5-aza-C) and 5-azadeoxycytidine (5-aza-CdR) (3). These nucleoside analogs affect many cellular processes and have been shown to alter cellular differentiation even in organisms that do not bear methylated bases in their genomes (4). 5-Aza-C is mutagenic and causes DNA damage in fission yeast (5) and in \textit{Escherichia coli} (6). To exert their biological effects, 5-aza-C and 5-aza-CdR must be incorporated into the DNA where they covalently trap the bulky 190-kDa DNA methyltransferase enzyme onto the DNA (7). The covalent trapping of DNA MeTase to the DNA and not hypomethylation of DNA itself is reported to be the cause of the mutagenic and cytotoxic effects of 5-aza-CdR (7). Experiments in which the DNA MeTase has been inhibited by genetic means, for example, by targeted disruption of the DNA MeTase gene in mice, resulted in directly opposite effects on gene expression to those observed with 5-aza-CdR treatment (8–10). Therefore, 5-aza-C and 5-aza-CdR may alter gene expression by mechanisms unrelated to the inhibition of DNA MeTase; for example, by modifying chromatin structure, as has been suggested (11–13). In fact, inhibition of the histone deacetylases, enzymes known to modify chromatin structure, by trichostatin A, has recently been found to reverse methylation-dependent transcriptional silencing (14, 15). This has prompted the suggestion that much of the transcriptional repression seen is due to histone acetylation and has reopened questions on the role of DNA MeTase in transcriptional repression (16).

In addition to the potential role the DNA MeTase plays in gene expression, it is also implicated in oncogenesis. Elevated levels of DNA MeTase activity have been observed in many cancer cells \textit{in vitro} (17) and tumors \textit{in vivo} (18, 19). Activation of the oncogenic ras signal transduction pathway has been shown to induce DNA MeTase expression (20, 21). In addition, elevated DNA MeTase levels are required to maintain the phenotype of these ras-transformed cells, suggesting that DNA methyltransferase is an important downstream effector of these pathways (22). This is supported by the recent finding that increased DNA MeTase levels are required to maintain the phenotype of fibroblasts transformed with the fos oncogene (23).

Thus, to isolate the role of the DNA MeTase itself in gene expression and cancer and to dissociate it from effects on chromatin structure requires specific DNA MeTase inhibitors that are not incorporated into genomic DNA. To this aim we have developed potent antisense inhibitors capable of specifically reducing cellular DNA MeTase levels and have employed these inhibitors to study the response of cancer cells to reduction of cellular DNA MeTase levels.

EXPERIMENTAL PROCEDURES

Oligonucleotide Treatment of Cells in Culture—Cells were treated with the 4×4 hybrid 2′-O-methylphosphorothioate antisense oligonucleotides; MG88 sequence (5′-AACGATGAGCCGGTCTCC-3′), where bold nucleotides are 2′-O-methyl modified) and MG208 (5′-AACGATCCGCCCTTGTCC-3′) at doses from 0 (Lipofectin alone) to 80 nM in the presence of Lipofectin (6.25 μg/ml). The cells were incubated...
for 24 h in complete medium. For longer treatments (2–10 days) the cells were transfected with oligonucleotides every day and split every second day.

**Western Blotting**—Whole cell lysates and nuclear extracts were prepared as described (51). The antibodies used were as follows: monoclonal antibodies p16\(^{INK4a}\) and retinoblastoma (Rb) from Pharmingen, monoclonal antibody Cip1 (p21\(^{WAF1/CIP1}\)) from Transduction Laboratories, affinity-purified polyclonal antibodies actin(l-19) from Santa Cruz, and MET TB antibody raised against glutathione affinity purified polyclonal antibodies actin(l-19) from Santa Cruz, and MET TB antibody to control for specificity of inhibition and protein loading.

**RESULTS AND DISCUSSION**

**Sequence-specific Reduction of Human DNA Methytransferase Protein Levels in Cancer Cells by DNA Methytransferase Antisense Inhibitors**—To identify antisense oligodeoxynucleotides capable of inhibiting DNA MeTase gene expression in human tumor cells, 85 phosphorothioate oligodeoxynucleotides (20 bases in length) bearing sequences complementary to the 5’ and 3’ regions of the human DNA MeTase mRNA as well as oligonucleotides targeted to intron-exon boundaries were synthesized and screened for antisense activity (Fig. 1A). Antisense oligodeoxynucleotides have been shown to act through an RNase H-dependent cleavage of the target mRNA (24, 25), which then following turnover of previously synthesized protein leads to a reduction in target protein. Two DNA MeTase mRNA regions highly sensitive to antisense inhibition were identified in this screen. These potent DNA MeTase antisense inhibitors, MG88 and MG98, are termed second generation antisense molecules because they contain both phosphorothioate backbone modifications as well as a 2’-O-methyl modifications to the ribose on the four 3’ and 5’ terminal nucleotides. The combination of these chemistries increases stability and potency of the inhibitors, thus allowing very low (nanomolar) concentrations to be used experimentally, thus minimizing nonspecific effects. MG88 and MG98 have IC\(_{50}\) values of 40 and 45 nM for inhibition of DNA MeTase protein, respectively. Both inhibitors demonstrated sequence-dependent inhibition of the DNA MeTase as scrambled and mismatch, control oligodeoxynucleotides of either one had no effect on DNA MeTase levels.

In the experiments presented here we focused on the DNA MeTase antisense inhibitor MG88. Treatment of T24 human bladder cancer cells and A549 human non-small cell lung can-
cancer cells with 0–80 nM antisense inhibitor MG88, a 20-base second generation chemistry phosphorothioate oligonucleotide targeted to the DNA MeTase 5’ region for 48 h, produced dose-dependent reduction in DNA MeTase protein levels (Fig. 1B). Treatment with the mismatch control oligomer MG208 (a 20-base second generation oligonucleotide with the same sequence as MG88 except 6 mismatched bases) or with Lipofectin alone produced no inhibition of DNA MeTase (Fig. 1B). The non-target protein, α-actin, was used as a control for protein loading (Fig. 1B). Several other human tumor cell lines, including the breast cancer lines MDA-MB231, MCF-7, the lung cancer cell line, H446, and the colon cancer cell lines HCT116, SW48, and LoVo were used to evaluate the activities of MG88 and MG208 with essentially identical results (data not shown).

DNA MeTase Protein Levels Control p16ink4A Expression by Methylation of Its Promoter Region—The cyclin-dependent kinase inhibitor (CDK) p16ink4A regulates the transition from G1 to S-phases of the cell cycle (26). Inactivation of p16ink4A is one of the most frequently observed abnormalities in human cancer (26). Genetic alterations in p16ink4A including point mutations (27–29) and to a greater extent homozygous deletion (30) are often found in tumors. Transcriptional inactivation and associated hypermethylation of the p16ink4A promoter region have also been observed in virtually all types of cancer (31–34). Treatment of cells with toxic doses of 5-aza-CdR can cause demethylation and induction of p16ink4A mRNA, detectable by reverse transcriptase-PCR, after recovery from the immediate toxic effects of this drug (31, 35, 36). To investigate the effect of specifically reducing cellular DNA MeTase levels on the expression and methylation status of a silenced p16ink4A gene, we treated the T24 human bladder cancer cells that contain a hypermethylated and silenced p16ink4A gene, with the human DNA MeTase inhibitor MG88. Re-expression of p16ink4A protein was detected after 5 days of treatment with either 40 or 75 nM MG88 (Fig. 2A). The latency of the reactivation is expected as inhibition of any molecular target (DNA MeTase in this case) by antisense requires first, that the target mRNA levels are reduced and then that turnover of previously synthesized protein is completed, in contrast to small molecule inhibitors of proteins that directly inhibit the target enzyme. In addition, in the absence of any active demethylating activity, demethylation by decreased methylation capacity is a passive process that requires DNA replication. p16ink4A reactivation was both dose-dependent and time-dependent (Fig. 2A). Due to the antiproliferative effect of MG88 itself (see Fig. 3C), p16ink4A levels were normalized to cell number (Fig. 2A, graph). p16ink4A was not detected in cells treated with either 40 or 75 nM of the mismatch control MG208 or Lipofectin alone (Fig. 2A). As expected, cellular DNA MeTase levels were reduced by MG88 but not by MG208 (Fig. 2A, lower panel).

Reactivation of p16ink4A by MG88 Causes Accumulation of Hypophosphorylated pRb and Inhibition of Cellular Proliferation—p16ink4A regulates progression through the G1 phase of the cell cycle by inhibiting cyclin-dependent kinase CDK4-mediated phosphorylation of pRb such that the hypophosphorylated form of Rb is associated with G1/G0 growth arrest (26). Reactivation of p16ink4A by MG88 treatment caused decrease in the phosphorylated forms of pRb, thus increasing the relative abundance of hypophosphorylated form of pRb over phosphorylated forms of pRb, while treatment of cells with either Lipofectin alone or the control MG208 did not alter the phosphorylation of pRb (Fig. 2D). These results demonstrate that high levels of DNA MeTase in T24 cells actively suppresses p16ink4A gene expression and that inhibition of DNA MeTase restores functional p16ink4A expression capable of regulating downstream molecular targets, such as pRb.

It is not known whether the DNA MeTase enzyme targeted by MG88 (Dnmt1) encodes only maintenance DNA methyltransferase activity or de novo methylation activity as well (37–40). To determine whether de novo methylation and silencing of the re-expressed p16ink4A gene occurs when DNA MeTase returns to control levels, treatments were stopped after 10 days and p16ink4A and DNA MeTase protein levels were determined on days 3, 5, and 7 post-treatment. High dose MG88 treatment (75 nM) reduced cell numbers by inhibition of proliferation and resulted in cell death after day 10 of treatment, therefore analysis of the duration of p16ink4A expression was restricted to MG88 40 nM treatments. DNA MeTase protein levels increased as expected in the absence of MG88 treatment and returned to control levels between days 5–7 post-treatment (Fig. 2C, middle panel). p16ink4A protein expression decreased steadily over the post-treatment period until it was barely detectable at day 7 post-treatment (Fig. 2B, upper panel). Fig. 2D shows the inverse relationship between DNA MeTase levels and p16ink4A levels during and after the treatment period. Of note is the fact that loss of p16ink4A expression begins at day 14 after DNA MeTase has returned to near control levels. This lag suggests that elevated levels of DNA MeTase over several rounds of replication are required to methylate and inactivate p16ink4A gene expression. That the inactivation and de novo methylation of p16ink4A observed are coincident with elevated levels of the DNA MeTase (Dnmt1) suggests that it may contribute to de novo methylation activity itself.

To identify changes in the methylation status of the p16ink4A promoter induced by MG88 treatment we performed MSP (41) and bisulfite genomic sequencing (42). MSP analysis revealed that demethylation of the p16ink4A promoter region occurred as early as day 3 of MG88 treatment (Fig. 3A). Treatment with MG208 or Lipofectin alone had no effect on methylation of the p16ink4A gene (Fig. 3A). Employing bisulfite genomic sequencing provided a more detailed dissection of the demethylation events at the p16ink4A promoter. Analysis of several clones for each treatment condition revealed that 15 CpG sites within the p16ink4A promoter are methylated in untreated T24 cells (Fig. 3B). Inhibition of the DNA MeTase by MG88 led to demethylation at 5 of 15 CpG sites by day 3 and demethylation at all 15 CpG sites by day 5 of treatment, whereas treatment with the control MG208 had no effect on p16ink4A methylation status (Fig. 3B). Three days after cessation of MG88 treatment the p16ink4A promoter shows significant re-methylation at 13 of 15 sites reflecting either de novo methylation of these sites or a rapid expansion of a less affected population (Fig. 3B).

To study the effect of specific inhibition of DNA MeTase on cell growth we monitored cellular proliferation rates both during the treatment and post-treatment periods to determine the duration of the effect. During the course of treatment MG88 dramatically inhibited cell proliferation, whereas treatment of cells with the control MG208 caused only minimal growth inhibition relative to Lipofectin-treated cells (Fig. 3C, panel I). Inhibition of cell proliferation persisted for approximately 1 week post-treatment, consistent with the finding that p16ink4A expression was maintained until 7 days after the last dose of MG88 (Fig. 2C). To determine whether the loss of p16ink4A expression after reactivation by short term treatment with MG88 was due to the proliferation of a less affected (less demethylated) population of cells within the treated population, or to rapid inactivation of p16ink4A after MG88 withdrawal, we isolated single cell clones after treatment. Several MG88 clones were, in fact, p16ink4A negative (data not shown), confirming as expected that MG88 treatment produce a mixed population of p16ink4A positive and negative cells. Isolation and methylation analysis of a MG88-treated p16ink4A expressing
Fig. 2. Effect of DNA MeTase inhibition on p16\textsuperscript{ink4A} protein expression and Rb phosphorylation in T24 cells. A, p16\textsuperscript{ink4A} protein levels were determined by immunoprecipitation Western analysis in T24 cells treated with Lipofectin only (transfection control), MG88 (DNA MeTase antisense), MG208 (mismatch control), at 40 or 75 nM for 3, 5, 8, or 10 days. HeLa cell served as the positive (+) control for p16\textsuperscript{ink4A}. Lower panel shows a Western blot for the DNA MeTase levels. Graph shows p16\textsuperscript{ink4A} levels normalized to cell number. B, phosphorylation of Rb protein was determined by Western blot with an antibody recognizing all phosphorylated forms of Rb. Rb phosphorylation in T24 cells was reduced in MG88-treated cells most significantly at the higher dose (75 nM). C, p16\textsuperscript{ink4A} and DNA MeTase post-treatment levels. p16\textsuperscript{ink4A} protein levels were determined by Western blot on whole cell lysates 3, 5, and 7 days after treatments were stopped. On day 3 post-treatment, p16\textsuperscript{ink4A} protein dropped to almost undetectable levels 7 days after MG88 treatment. DNA MeTase protein levels rose in the absence of MG88 and returned to control levels between days 5 and 7 post-treatment. D, graph shows the quantitation of DNA MeTase and p16\textsuperscript{ink4A} levels during the course of MG88 treatment and post-treatment periods.
FIG. 3. Effect of DNA MeTase inhibition on DNA methylation by MSP, bisulfite sequencing, and cell growth. 

**A,** methylation-specific PCR (28) of the p16ink4A promoter was performed on treated T24 cells at the indicated days. PCR primers specific for methylated p16ink4A (M) and unmethylated p16ink4A (U) show that demethylation occurred in MG88-treated cells only, as early as day 3. **B,** bisulfite genomic sequencing results of the p16ink4A proximal promoter. Schematic summarizing p16ink4A demethylation events over the treatment time course and post-treatment in MG88-treated T24 cells, MG208 treatment had no effect on p16ink4A promoter methylation (data not shown). **C:** panel 1, growth curve of T24 cells during treatment (days 0–5) and post-treatment (days 5–18). Panel 2, growth curve of single cell clones isolated after a 5-day treatment with MG88 (MGC4-5), MG208 (MG208C2-5), or Lipofectin (MGlipoC5). Clones were cultured for 35 days post-treatment when proliferation assay was initiated. Proliferation of MG88C4-5 was slow compared with control clones (MG208C2-4, Lipofectin MGlipoC5) from days 40 to 45 post-treatment when growth rate increased rapidly. Cell numbers at day 49 post-treatment are shown at the far right of the graph in brackets. p16ink4A protein levels in MG88C4-5 (inset) at post-treatment days 36 and 49. **D,** bisulfite sequencing of p16ink4A promoter in clone MG88C4-5 at day 30 post-treatment, demethylation of CpG sites is indicated by arrows.
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A clone (MG88C4-5) revealed that the p16\(^{ink4A}\) promoter region was completely non-methylated at all CpG sites evaluated even after 30 days in culture post-MG88 treatment (Fig. 3D). Thus demonstrating that even short-term (5 day) inhibition of the DNA MeTase by MG88 could induce sustained re-expression of a silenced tumor suppressor gene. Clone MG88C4-5 grew very slowly compared with clones isolated after Lipofectin or MG208 treatment (Fig. 3C, panel 2); however, after 40–45 days in culture post-treatment, the growth rate of MG88C4-5 cells increased dramatically (Fig. 3C, panel 2). Determination of p16\(^{ink4A}\) protein levels in MG88C4-5 cells revealed a significant decrease at this time point (Fig. 3C, inset). Loss of p16\(^{ink4A}\) expression after prolonged culture in the absence of MG88 treatment suggests that the DNA MeTase targeted (thought to encode maintenance DNA MeTase activity) may have de novo methyltransferase activity and over time can methylate and inactivate previously unmethylated actively expressing genes.

The antiproliferative effect of DNA MeTase inhibition on T24 cells was apparent as early as 48 h after the first treatment (Fig. 3C), although p16\(^{ink4A}\) expression was only detected after day 5 of treatment (Fig. 2A). Thus, p16\(^{ink4A}\) reactivation alone cannot explain the inhibition of proliferation observed.

**Rapid Induction of p21\(^{WAF1}\) by DNA MeTase Inhibition**—Another member of the cyclin-dependent kinase inhibitor (CDKI) family p21\(^{WAF1}\), inhibits a wide range of cyclin-CDK complexes involved in G\(_1\) and S phase progression (43–45). Recently it has been observed that an inverse correlation exists between p21\(^{WAF1}\) and DNA MeTase protein levels in SV40-transformed and nontransformed cells (46). In addition, p21\(^{WAF1}\) and the DNA MeTase have recently been shown to compete with each other for binding to proliferating cell nuclear antigen (PCNA) (46). To investigate whether DNA MeTase and p21\(^{WAF1}\) protein levels are linked by a regulatory pathway, we determined p21\(^{WAF1}\) protein levels in untreated T24 cells and in T24 cells in which DNA MeTase levels had been incrementally reduced by MG88 treatment. p21\(^{WAF1}\) increased directly with the reduction in DNA MeTase (Fig. 4, A and B), while neither Lipofectin nor MG208 had an effect on either DNA MeTase or p21\(^{WAF1}\) levels (Fig. 4, A and B). DNA MeTase inhibition induced p21\(^{WAF1}\) in a dose-dependent fashion as early as 24 h after MG88 treatment (Fig. 4B), consistent with a role for p21\(^{WAF1}\) in the antiproliferative effect observed (Fig. 3C). These findings demonstrate that a functional antagonism between DNA MeTase and p21\(^{WAF1}\) on cellular proliferation exists. Furthermore, these results provide evidence that the DNA MeTase plays a direct role in cancer cell proliferation. It has been argued that DNA MeTase should not be involved in proliferation as embryonic stem cells from mice homozygous for Dnmt1 mutation (DNA MeTase knock out) proliferate normally (47). However, Dnmt1\(^{-/-}\) embryonic stem cells die upon differentiation (47), death of these cells, however, can be rescued by the expression of a DNA MeTase isofrom expressed from the same Dnmt1 gene (48). Although Dnmt1\(^{-/-}\) embryonic stem cells proliferate normally, Dnmt1\(^{-/-}\) mice die at mid-gestation (49). Phenotypic and histological analysis of Dnmt1\(^{-/-}\) embryos revealed reduced cell proliferation and widespread cell death (49), supporting a role for DNA MeTase in cell proliferation.

To determine if p21\(^{WAF1}\) was induced at the transcriptional level we performed RNAse protection assays on cells treated with 40 nM of either MG88 or MG208 for 24 and 48 h. No increase in p21\(^{WAF1}\) mRNA was seen in response to this treatment as demonstrated by RNAse protection analysis (Fig. 4C), suggesting that post-translational regulation of the p21\(^{WAF1}\) protein is involved. Given that p21\(^{WAF1}\) mRNA levels do not change with and that p21\(^{WAF1}\) protein levels rise rapidly even with minimal inhibition of the DNA MeTase (Fig. 4, A and B), DNA demethylation and transcriptional activation are not likely to be involved as a mechanism of p21\(^{WAF1}\) induction. p21\(^{WAF1}\) controls cell cycle transition at the G\(_1\)-S boundary.
by forming a complex with PCNA, cyclin D1, and CDK4 (46). Human DNA Methylase can compete with p21WAF1 for PCNA binding (46). This DNA Methylase-PCNA complex is found in transformed cells with high levels of DNA Methylase but not in non-transformed cells and can be disrupted by p21WAF1-derived peptides (50). This competition between DNA Methylase and p21WAF1 for the same binding site on PCNA may explain the inverse relationship and regulation observed. In such a model, high levels of DNA Methylase, as observed in cancer cells, would compete and displace p21WAF1 from its target PCNA, the free p21WAF1 may be more susceptible to proteolytic degradation than p21WAF1 in a complex with PCNA and other proteins. Conversely, higher levels of p21WAF1 would displace DNA Methylase from PCNA and lead to its degradation. Thus the inverse relationship and regulation observed. In such a downstream effect, DNA Methylase found in transformed cells may effectively regulate proliferation both by reducing cellular levels of DNA Methylase and by an alternate mechanism involving hypermethylation and transcriptional silencing of tumor suppressor genes (p16ink4A) and by an alternate mechanism, most likely mediated by protein-protein interactions, as in the case of p21WAF1. In addition, these findings suggest that non-nucleoside, non-incorporating direct inhibitors of the human DNA Methylase enzyme may be of value in the treatment of human cancer.

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