Modified Oligonucleotides as Bona Fide Antagonists of Proteins Interacting with DNA

HAIRPIN ANTAGONISTS OF THE HUMAN DNA METHYLTRANSFERASE*

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The study of the biological role of DNA methyltransferase (DNA MeTase) has been impeded by the lack of direct and specific inhibitors. This report describes the design of potent DNA based antagonists of DNA MeTase and their utilization to define the interactions of DNA MeTase with its substrate and to study its biological role. We demonstrate that the size, secondary structure, hemimethylation, and phosphorothioate modification strongly affect the antagonists interaction with DNA MeTase whereas base substitutions do not have a significant effect. To study whether DNA MeTase is critical for cellular transformation, human lung non-small carcinoma cells were treated with the DNA MeTase antagonists. Ex vivo, hairpin inhibitors of DNA MeTase are localized to the cell nucleus in lung cancer cells. They inhibit DNA MeTase, cell growth, and anchorage independent growth (an indicator of tumorigenesis in cell culture) in a dose-dependent manner. The inhibitors developed in this study are the first documented example of direct inhibitors of DNA MeTase in living cells and of modified oligonucleotides as bona fide antagonists of critical cellular proteins.

DNA-binding proteins that regulate gene expression play an important biological role and are potentially attractive therapeutic targets. However, the study of their role in different physiological and pathological processes has been hindered by the lack of specific inhibitors. These proteins are especially appealing as drug targets because their ligand is a DNA sequence that can be identified by standard molecular biology techniques and can be synthesized and modified by well established chemistries (1). In addition, recent observations suggest that double-stranded DNA-based oligonucleotides bearing transcription factor recognition sites can be delivered into cells in culture and in vivo and exhibit pharmacological effects (2, 3). A major limitation of this approach is that oligonucleotide antagonists that are identical to the transcription factors cognate site act as stochiometric competitors. Therefore, very high intracellular concentrations are required to effectively engage all the transcription factor available in the cell at all times. An ideal DNA-binding protein antagonist should exhibit higher affinity to the protein than the cognate sequence and bear a slow off rate. In this report we have tested the hypothesis that DNA-based inhibitors of DNA-binding proteins that address these requirements could be developed, using the DNA methyltransferase enzyme (DNA MeTase)1 as a model DNA-binding protein.

Basic oncogenic pathways such as the Ras-Jun signaling pathway have been shown to up-regulate DNA MeTase mRNA (4–6) and the hyperactivation of DNA MeTase observed in many cancer cells (7, 8) occurs in parallel with the development of the aberrant patterns of DNA methylation that these cells exhibit (9, 10). A number of studies suggest that the hyperactivation of DNA MeTase plays a causal role in oncogenesis. For example, the intraperitoneal injection of antisense oligonucleotide to DNA MeTase mRNA into LAF/1 mice bearing tumors derived from the syngeneic tumor cell line Y1 (11) inhibits tumor growth; and in vivo reduction of DNA MeTase levels by either 5-azaCdR treatment or by bearing one mutated allele of DNA MeTase reduces the frequency of appearance of intestinal adenomas in the Min mouse bearing a mutation in the adenomatosis polyposis coli gene (12).

Uncovering the biological role of DNA MeTase in vivo requires the availability of specific DNA MeTase inhibitors especially since mice bearing a null mutation of the DNA MeTase die at midgestation (13). The most established inhibitors of DNA MeTase are nucleoside analogs such as 5-fluorouracil and 5-azaCdR (14), which are believed to covalently trap the DNA MeTase after incorporation into DNA (15). It has been recently shown that this mechanism of action is responsible for the cytotoxic and mutagenic side effects of 5-azaCdR (16, 17), which seriously limits their ability to be used in either therapeutics or research (18). One direct inhibitor of DNA MeTase is S-adenosylhomocysteine (19); however, S-adenosylhomocysteine is not a specific inhibitor of DNA methylation and might inhibit other methylation reactions in the cell. We reasoned that oligonucleotide-based DNA analogs that bind the DNA MeTase but are not acceptors of the methyl group will form a high affinity stable intermediate with DNA MeTase and will be potent inhibitors of DNA MeTase. In this paper, we describe the design of self complementary oligonucleotides that form “hairpin” structures and demonstrate that a combination of modifications of the DNA sequence, methylation status, and phosphate backbone of these oligonucleotides results in modified substrates that act as potent and efficacious direct inhibitors of DNA MeTase.

1 The abbreviations used are: DNA MeTase, DNA methyltransferase; PAGE, polyacrylamide gel electrophoresis; AdoMet, S-adenosylmethionine.
**EXPERIMENTAL PROCEDURES**

**Oligonucleotide Synthesis—**All oligonucleotides were synthesized at Hybricon Inc. using standard phosphoramidite chemistry as described previously, purified on a 20% denaturing PAGE, eluted by salt extraction, denatured by boiling, and reannealed by slow cooling to room temperature, and dissolved in water at a final concentration of 1 mM.

**Cell Culture—**A549 non-small cell lung carcinoma cells (ATCC, CCL 185) and Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) and 4 ml of DPTI-SEM serum-free medium and left at room temperature for 15 min and then added to the cells. The hairpin containing medium was removed from the cells and replaced with regular growth medium after 4 h. The treatment was repeated 3 times at 24-h intervals. The cells were split after the third treatment and equal numbers of cells were plated on 10-cm tissue culture plates and harvested after 24 h.

**DNA MeTase Activity Assay—**DNA MeTase activity was assayed using nuclear extracts (5 μg of total protein) prepared from human lung carcinoma cell line H446 as described previously (20). The reaction mixture (final volume of 30 μl) included the indicated concentrations of oligonucleotides in a buffer containing 10 mM Tris-HCl, 25% glycerol, 5 mM MgCl₂, 0.5 mM phenylmethylsulfonyl fluoride, 0.15 mM NaCl, 1 μl of S'-methyl-32H]-adenosyl-1-methionine (7.5 μCi/ml, Amersham) (AdoMet) as a methyl donor, and 0.2 μM of a synthetic hemimethylated double-stranded oligonucleotide substrate as a methyl acceptor as described previously (20). Following 2 h incubation at 37 °C the reaction was inactivated by heating at 65°C for 5 min and the incorporation of methyl groups into the DNA substrate was determined by scintillation counting of trichloroacetic acid precipitable counts. For each experiment, the background counts reflecting methylation of other macromolecules present in the nuclear extract as well as endogenous DNA methylation were determined by incubating the nuclear extract under the same conditions in the absence of the hemimethylated substrate. In a typical experiment the background counts obtained with nuclear extract alone were around 3000 dpm. The background counts were subtracted from the values obtained per each point. The DNA methylation transferase reaction is linear for 3 h under these conditions. The results are presented as an average of three determinations and the apparent constant of inhibition was determined by curve fitting of the dose-response curve using Sigma Plot software.

**Purification of DNA MeTase from A549 Cells by Chromatography—** DNA MeTase was purified from A549 human lung carcinoma cells. Nuclear extracts were prepared from cultures at near confluence. Nuclei were isolated by resuspending and incubating the cells in buffer A (100 mM Tris, pH 8.0, 0.5 mM MgCl₂, 5 mM KCl, and 0.5% Nonidet P-40) for 10 min, washed twice by centrifugation at 1,000 × g for 10 min. The nuclear pellet were resuspended in buffer A (400 μl) and collected as above. Nuclear proteins were extracted by resuspending and incubating the nuclear pellet in buffer B (30 μl/10 cells; 20 mM Tris, pH 8.0, 0.25% glycerol, 0.2 mM EDTA, and 0.4 mM NaCl) at 4°C for 15 min, followed by centrifugation at 10,000 × g for 30 min to separate the nuclear extract from the supernatant from the chromatin pellet. Nuclear extracts were stored at −80 °C. Freshly prepared nuclear extract was dialyzed to a conductivity equivalent to 0.2 M NaCl and applied onto a DEAE-Sepharose (Pharmacia) column (1.0 × 5 cm), which was pre-equilibrated with buffer P (10 mM potassium phosphate, pH 7.5, 1 mM sodium EDTA, 14 mM β-mercaptoethanol, 10% glycerol) containing 0.2 M NaCl. The column was operated at a flow rate of 1 ml/min. After sample loading, the column was washed withbuffer P and then starting buffer (buffer P + 0.2 M NaCl) and the proteins were eluted with 5 ml of a linear NaCl gradient (0.2–1.0 M). 0.5-ml fractions were collected and assayed for DNA MeTase activity as described above after desalting through a Microcon 10 (Amicon Inc.) spin column. DNA MeTase eluted between 0.3 and 0.5 M NaCl. The pooled active fractions were adjusted to 0.2 M NaCl by dialysis and applied onto a Q-Sepharose column which had been pre-equilibrated with buffer P at a flow rate of 1 ml/min. The column was washed and eluted with a 5 ml of a linear NaCl gradient of 0.2–1.0 M. 0.5-ml fractions were collected and assayed for DNA MeTase activity after desalting and concentrating (to a final volume of 0.2 ml) through a Microcon 10 spin column. MeTase activity eluted between 0.3 and 0.4 M NaCl. The pooled active fractions were adjusted to 0.2 M NaCl, loaded onto a 2.0 × 2.0 cm DEAE-Sephael (Pharmacia) column, and eluted with 10 ml of buffer P containing 0.2 M NaCl. The fractions (0.8 ml) were collected and assayed after concentration to about 200 μl for MeTase activity. Activity was detected at fraction 4, which is very near the void volume (Table I). A silver staining PAGE analysis revealed one distinct band migrating around 200 kDa. DNA MeTase is an unstable protein in our hands which explains in part the loss of activity through the purification procedure (Table I). This loss of activity can explain why the fold purification as determined by enzymatic assays is only 240-fold whereas a single distinct band is identified by a silver-stain PAGE analysis. The identity of the band as DNA MeTase was verified by a Western blot analysis of the purified fraction with a previously described anti DNA MeTase polyclonal antibody (11).

**Purification of Recombinant Human DNA MeTase—** The cDNA bearing the coding sequences of human DNA MeTase was generated as follows. Subdomains of the human DNA-cytosine-5-methyltransferase cDNA were generated by reverse transcriptase-polymerase chain reaction from 1 μg of total RNA prepared from the human small lung carcinoma cell line H446 using the following set of primers: a, 5'-ccctaggggtgcgcaagaaa-3' (sense); 163–190 and 5'-gcagccctgctc-3' (antisense); 611–592; b, 5'-tattcgcagggaggtctact-3' (sense); 454–473 and 5'-ccctcctcccttcagggcctc-3' (antisense); 3573–3551; c, 5'-ggagggaggagggacgcg-3' (sense); 3571–3590 and 5'-gcttcagcttcctc-3' (antisense); 5085–5066. The nucleotide positions are according to accession number X63892. The polymerase chain reaction products were cloned in pCR3.1 vector (Invitrogen) and the sequence of the cDNA was verified by dideoxy chain termination method using a T7 DNA sequencing kit (Pharmacia) and alignment to the published human DNA MeTase sequence (21). To generate the DNA MeTase construct we first cleaved the pCR 3.1 bearing fragment c with EcoRI, the fragment was blunted and ligated to a pCR3.1 vector bearing fragment b which was cleaved at the 3' EcoRV site. This construct was then cleaved with XhoI and ligated to a pcR3.1 vector bearing fragment a which was cleaved at an internal XhoI site to form the reassembled MeTase construct. This plasmid can direct expression from the bacteriophage T7 or the mammalian cytomegalovirus promoter. *Escherichia coli* BL21 DE 3 cells (expressing T7 polymerase under a Lac promoter) were transformed with plasmid pCR3.1MeTase and grown in 2 liters of Luria broth medium at 37 °C up to an OD₆₀₀ of 0.6. The cells were induced with 1 mM isopropyl-β-D-1-galactopyranoside (IPTG) for 6 h. Following centrifugation at 4000 rpm for 10 min, the cells (10 g) were suspended in 10 ml of buffer P, sonicated for 5 min with a burst and a gap of 15 s. The supernatant was separated by centrifugation at 4000 rpm for 20 min and was blended with the protease inhibitors aprotinin, leupeptin, and Pefabloc SC (Boehringer-Mannheim) (10 μl each of 1 mg/ml stock solution) and loaded onto a 10-ml phosphocellulose column equilibrated with Buffer P + 0.2 M NaCl. Active fractions were identified by a DNA MeTase assay as described above and were pooled and further purified by Q-Sepharose and DEAE-Sepharose chromatography as described above for A549 human lung MeTase.

**Electrophoretic Mobility Shift Assay—** To identify DNA-protein complexes formed between DNA MeTase and the hairpin inhibitors, an electrophoretic mobility shift assay was performed. The different oligonucleotides were labeled at their 5' with ³²P by incubating 5 μM purified oligonucleotide with 50 μCi of [³²P]ATP (3000 Ci/mmol, Amersham) and 10 units of T4 polynucleotide kinase (Boehringer-Mannheim) in a final volume of 50 μl at 37 °C for 2 h. The labeled oligonucleotide was then purified on a 6% denaturing PAGE to remove the T4 polynucleotide kinase and the unincorporated [³²P]ATP, eluted from the gel, ethanol precipitated, and resuspended in double-distilled water. This mixture was heated at 90 °C for 5 min and the hairpin was annealed by allowing the mixture to cool slowly to 25°C.

The DNA binding reactions contained 20 mM HEPES (pH 7.5), 12% glycerol, 1 mM EDTA, 4 mM dithiothreitol, 0.1% Nonidet P-40, 3 mM MgCl₂, 1 μg of purified human MeTase, 5 μg of bovine serum albumin, 300 μM scrambled phosphorothioate oligonucleotide as a non-specific control.
competing (5'-CGATTTAATCCCTAACCTCTC), and the indicated concentration of hairpin oligonucleotide in a final reaction volume of 10 µl. The mixtures were incubated for 30 min at room temperature, and the complexes were resolved by electrophoresis in a 4% non-denaturing polyacrylamide gel at 4 °C for approximately 3 h at 220 volts. For competition assays, a 100-fold molar excess of the double-stranded oligonucleotide was added to the reaction mixture prior to adding the labeled test hairpin inhibitor.

For determination of apparent dissociation constants (Kd), the level of complex formation at different substrate concentrations was quantified with a PhosphorImager and plotted against the substrate concentration. The Kd was calculated using the Sigma plot software. To determine the stability of the complex formed with the hemimethylated hairpin (Kdθ), a binding reaction mixture of 100 µl was prepared with the same final concentrations described above. After an initial incubation time of 30 min, 100-fold excess of competitor was added (time 0) at room temperature and 10 µl of the binding reaction were removed at each time point and loaded on a 5% non-denaturing polyacrylamide gel.

**RESULTS**

**Design Principles**—Our general goal was to design a modified DNA MeTase substrate that will bind the DNA MeTase with higher affinity than the unmodified sequence, will not serve as a methyl acceptor in the reaction, but will occupy the enzyme. The following principles were used in our initial design of modified substrates. First, the preferable substrate for vertebrate DNA MeTase is a hemimethylated CpG dinucleotide contained in double-stranded DNA (23). However, it stands to reason that coinhibition in vivo of two annealed oligonucleotides without disturbing their interaction is an arduous task. There is documented evidence, however, that single-stranded oligonucleotides could be delivered ex vivo and in vivo (2). We therefore opted at designing self-complementary single-stranded oligonucleotides that can form hairpin structures and enable the formation of a hemimethylated duplex (see Table II for prototype structures). A hemimethylated DNA duplex forms, however, only a low affinity complex with mammalian DNA MeTase (24, 25). It is therefore clear that certain modifications will be required to generate a potent inhibitor of DNA MeTase. Altering the recognition sequence in the hemimethylated CpG dinucleotide pair can significantly alter the affinity of the enzyme to the substrate and the rate of methylation (26). For example, it has been shown that structures associated with more weakly stacked cytosine rings, such as mispaired or abasic duplexes and other unusual DNA structures, are preferentially methylated by the human DNA MeTase (26, 27).

We designed our hairpins to recapitulate the natural substrate. The 5’ arm of the hairpin bearing methylated CpGs mimics the parental methylation guiding strand whereas the 3’ arm of the hairpin mimics the nascent methyl-acceptor strand of replicating DNA (Table II). As our goal was to design an inhibitor of DNA MeTase, we tested the hypothesis that introducing changes similar to those previously described (26, 27) to the methyl acceptor strand will increase the time of occupancy of the enzyme by the substrate but as well as inhibit methyl transfer. In addition, very little is known about the interactions of the DNA MeTase and the phosphate and sugar components of the backbone. We reasoned that these interactions might be critical in determining the ability of the enzyme to interact with the substrate as well as its ability to catalyze the methyl transfer.

**Modifications of the Phosphate Backbone and the State of Methylation Strongly Affect the Potency of Hairpin DNA MeTase Inhibitors**—Based on these principles, a set of single-stranded oligonucleotides to act as substrates for DNA MeTase were synthesized (Tables II and III). Both hemimethylated and nonmethylated variants were synthesized as well as various substitutions to the C located opposite the methylated CpG dinucleotide. The two CpG sites in each oligonucleotide were separated by 4 bases to avoid tandem CpGs which were previously shown to be poor substrates of DNA MeTase (28). All compounds are expected to form a hairpin structure based on previously documented published data and theoretical considerations (29–31). We have verified that these oligonucleotides form double-stranded structures at 37 °C (data not shown). The potency of the oligonucleotides as inhibitors of DNA MeTase was determined by measuring the rate of DNA MeTase-catalyzed transfer of a tritiated methyl group from S-adenosylmethionine to a standard hemimethylated double-stranded oligonucleotide substrate (20 in a nuclear extract prepared from a
human small cell lung carcinoma line H446 in the presence of increasing concentrations of these oligonucleotides. Typical curves are presented in Fig. 1. The ability of the inhibitors to serve as acceptors of DNA MeTase-catalyzed transfer of methyl groups in the absence of the standard substrate was determined using saturating concentrations of the inhibitors (1 μM).

Nuclear extracts were used rather than purified enzyme to recapitulate the situation in vivo where DNA MeTase functions in the presence of other nuclear proteins and to ascertain that the inhibitors are effective in the nuclear milieu.

The following conclusions, regarding the structure function relationship of DNA MeTase antagonists, were derived from the screening of the different DNA MeTase substrates shown in Table II. First, a non-modified hemimethylated hairpin oligonucleotide (3048) is a poor inhibitor of DNA MeTase demonstrating, as predicted in the introduction, that competitive inhibition using a cognate site of a DNA-binding protein is inadequate. Second, modification of the recognition sequence of DNA MeTase, by altering one methyl acceptor CpG site to inosine (IpG) and removing its methyl acceptor capacity, does not increase the potency of the inhibitor (3046). Third, modifying the hairpin phosphate backbone by replacing one of the oxygen groups with a thiol group (3016, 3018) abolishes its methyl acceptor capacity and results in potent inhibition (Ki values at the 30–65 nM range). Fourth, hemimethylation increases the potency of hairpin inhibitors (Table III and Fig. 1; 3016, 3018 versus 3056, 3093). Hemimethylation of a single 5' site resulted in intermediate potency of the inhibitor (3050 versus 3018 and 3056), suggesting that methylation of the second site in the upper arm of the hairpin affects the affinity of the MeTase to the substrate. Fifth, a cognate methyl CpG dinucleotide is required opposite the methyl acceptor site (Table III, 3044 versus 3017) whereas thymidine is adequate in the second 3'-methyl guiding site (Table III, 3019 versus 3016) as has previously been suggested (26). Sixth, surprisingly, a fully methylated phosphorothioate hairpin (3191) is a potent inhibitor of DNA MeTase, suggesting high affinity recognition of a CpG site which is methylated on both strands by the DNA
MeTase (Table III). Seventh, methylation at cytosines found in CpC dinucleotides located upstream to the CpG dinucleotide (3092) enhances 4-fold the potency of the inhibitor relative to the unmethylated counterpart (3093) suggesting that the DNA MeTase can recognize noncanonical methylated cytosines. Eighth, backbone modifications affect the interaction of DNA MeTase with the methyl acceptor site since phosphorothioate modification of 9 bases in the 3'-methyl acceptor arm of a hemimethylated hairpin (3062) is sufficient to confer high affinity antagonism of DNA MeTase whereas phosphorothioate modification of the methyl acceptor arm that does not include the first methyl acceptor site (3061 and 3063) is inadequate. Ninth, modification of the sugar moiety by 2'-O-methylation (3060 versus 3016) abolishes both the DNA MeTase inhibitory as well as methyl-acceptor activity suggesting an interaction between the DNA MeTase and the deoxyribose component of DNA. Alternatively, it is possible that the ribose-0-methyl groups indirectly affect MeTase-oligo interaction. Tenth, substituting the methyl acceptor residue to either uracil (3017), or inosine (3018 and 3062), or a fluoro substitution (3005), does not significantly reduce or increase its potency as an inhibitor. However, an abasic site (3053) reduces the potency of inhibition ($K_i$ of 350 nM). In summary, this structure-activity relationship study suggests that the interaction of DNA MeTase with the methyl acceptor site is influenced by the phosphate backbone, the presence of a hairpin, and its methylation state but not by the identity of the acceptor base. This study has identified potent inhibitors of DNA MeTase as well as nonactive analogs.

**Table III**

| Compound | Apparent $K_i$ | Methylation % of control |
|----------|----------------|--------------------------|
| 5' CTAACG*GATTC  | 3016, PS  | 65 nM  | 1.5 |
| 3' GACCCG*TACG  | 3017, PS  | 30 nM  | 10  |
| 5' CTAACG*GATTC  | 3018, PS  | 30 nM  | 9.3 |
| 3' GACCCG*TACG  | 3033, PS  | 350 nM | 11.4 |
| 5' CTAACG*GATTC  | 3005, PS  | 50 nM  | 7.7 |
| 3' GACCCG*TACG  | 3051, PS  | 350 nM | 0   |
| 5' CTAACG*GATTC  | 3052, PS  | 450 nM | 0   |
| 3' GACCCG*TACG  | 3019, PS  | 50 nM  | 15.3|
| 5' CTAACG*GATTC  | 3044, PS  | 650 nM | 8.0 |
| 3' GACCCG*TACG  | 3050, PS  | 350 nM | 5.3 |
| 5' CTAACG*GATTC  | 3191, PS  | 35 nM  | 2.8 |
| 3' GACCCG*TACG  | 3056, PS  | >1000 nM | 3 |
| 5' CTAACG*GATTC  | 3093, PS  | 400 nM | 2.8 |

DNA MeTase activity and the $K_i$ of the different hairpins was determined as described in Table II.

**DNA MeTase Hairpin Antagonists Specifically Inhibit DNA MeTase but Not Other Proteins Interacting with DNA—**To determine whether the active hemimethylated hairpin inhibits specifically mammalian DNA MeTase but not other DNA interacting proteins, we determined whether increasing concentrations of the active hairpin (3018) or the inactive hairpin (3060) will inhibit extension of primed DNA by DNA polymerase(s) in human nuclear extracts. As observed in Fig. 2A, there is only a small inhibition of DNA extension activity by the active 3018 hairpin in comparison with the inactive hairpin. The hairpins 3017, 3018, and 3044 do not specifically inhibit HpaII MeTase as measured by the ability of HpaII MeTase to confer resistance to HpaII cleavage. No inhibition of HpaII MeTase by hairpins 3017, 3018, and 3044 are observed up to 100 nM, a concentration at which 80–90% of mammalian DNA MeTase is inhibited (Fig. 2B). We redetermined the $K_i$ value of
were performed resulting in the calculated
not due to the absence of some component found in the nuclear
concentrations of the indicated hairpins (1–1000 nM) hemimethylated 3016,
activity by methylated, hemimethylated, nonmethylated and
extracts (5
m
K
II. The
S.D. The
K
Dose-response analysis of inhibition of DNA MeTase
hemimethylated 3060 hairpins, as described under “Experimental Pro-
nonmethylated 3093, fully methylated 3191, and 2’-O-methyl-modified
interactions with proteins present in the
in vitro
boiling (B
salt (1M NaCl) and that it is only eluted from the hairpin by
DNA MeTase is bound to the hairpin as indicated by its ab-
domain of DNA MeTase (Fig. 3

was reacted with an antibody directed against the catalytic
polymerase.
Eco
RI, or other DNA modifying proteins such as DNA
CpG MeTases such as
Hpa
II DNA MeTase. Similarly, these hairpins do
not inhibit EcoRI MeTase (data not shown). The data presented
here is consistent with the hypothesis that oligonucleotide 3018
specifically inhibits mammalian DNA MeTase but not other
 CpG MeTases such as
Hpa
II, other non-CpG MeTases such as
EcoRI, or other DNA modifying proteins such as DNA
polymerase.

Hairpin Inhibitors of DNA MeTase Form a Stable Complex
with the Enzyme—One possible mechanism explaining the in-
hibitory effect of the hairpin oligonucleotides described above is
that they stabilize a transition state complex with the enzyme.
Since they are not acceptors of a methyl group as shown above,
the enzyme is not removed from the substrate as it normally is
following a methyl transfer, and instead remains stably bound
to it. To test this hypothesis, we incubated a nuclear extract
prepared from human non-small cell lung carcinoma H446
with a hemimethylated hairpin (3018) that had a biotin moiety
at its 5’ end. The oligonucleotide-bound proteins were sepa-
rated from the unbound supernatant using avidin-coated mag-
netic beads. Following a wash with a high salt buffer, the
hairpin bound fraction was eluted from the hairpin bound
beads by boiling in an 0.1% SDS buffer. The different fractions
were subjected to a Western blot analysis and the membrane
was reacted with an antibody directed against the catalytic
domain of DNA MeTase (Fig. 3A). The results demonstrate that
DNA MeTase is bound to the hairpin as indicated by its ab-
sence in the supernatant fraction, that it is not eluted by high
salt (1 M NaCl) and that it is only eluted from the hairpin by
boiling (B). This data is consistent with the hypothesis that
DNA MeTase in nuclear extracts forms a stable noncovalent
complex with a hemimethylated hairpin oligonucleotide. To
verify that other nuclear proteins do not form similar stable
complexes with DNA MeTase, we reacted the membrane
against a retinoblastoma protein (Rb) specific antibody. As
observed in Fig. 3A, the Rb protein is fully washed away (W)
and none is stably bound to the hairpin as evidenced by its
absence in the boiled fraction (B).
To further ascertain that DNA MeTase stably binds hemi-
methylated hairpins, we in vitro transcribed and translated
human DNA MeTase in the presence of [35S]methionine and
reacted it with the biotinylated hairpin as described above. As
seen in Fig. 3A (TNT), the in vitro translated DNA MeTase is
stably bound to the hairpin as indicated by its elution in the
boiled fraction (B). To exclude the possibility that the binding of
the in vitro translated DNA MeTase reflects some unknown
interactions with proteins present in the in vitro translation
reaction mixture, we expressed recombinant DNA MeTase in
E. coli, purified it to homogeneity by chromatography as de-
scribed under “Experimental Procedures,” bound it to 32P-la-

![Graph](image)

FIG. 1. Dose-response analysis of inhibition of DNA MeTase activity by methylated, hemimethylated, nonmethylated and 2’-O-methyl-modified hairpins. DNA MeTase activity in nuclear extracts (5 µg) was determined in the presence of increasing concentra-
tions of the indicated hairpins (1–1000 nM) hemimethylated 3016, nonmethylated 3093, fully methylated 3191, and 2’-O-methyl-modified
hemimethylated 3060 hairpins, as described under “Experimental Pro-
ccedures.” The data presented is an average of three determinations ±
S.D. The
K
value obtained was similar (data not shown) indicat-
ing that 3016 interacts specifically with human DNA MeTase,

![Image](image)

FIG. 2. Hairpin inhibitors of DNA MeTase do not inhibit either
DNA extension activity in nuclear extracts or
Hpa
II MeTase. A, DNA polymerase activity. DNA extension activity was assayed in
nuclear extracts prepared from A549 cells using random primed SK-
plasmid as a primed template, [α-32P]dCTP and the other three dNTPs
in the presence of increasing concentrations (1–1000 nM) of the indi-
cated oligonucleotides, the active hairpin 3018 or the inactive hairpin
3060. A nonspecific phosphorothioate oligonucleotide sequence (Scr)
was used as a control for each concentration of oligonucleotide. The rate
of incorporation of labeled dCTP at each dose was determined, normal-
ized against the rate of incorporation of dCTP into DNA with the same
dose of nonspecific phosphorothioate oligonucleotide, and presented as
% of control. B, Hpa
II MeTase activity. SK- plasmid was incubated with
Hpa
II and the indicated concentrations of active hairpin (3017, 3018),
inactive hairpin (3044), and scrambled oligonucleotides. The Hpa
II-
methylated DNA was subjected to digestion with Hpa
II restriction
enzyme and the digestion products were subjected to Southern blot
analysis using 32P-labeled SK as a probe.
beled hemimethylated oligo 3016 and resolved the complex formed by nonadenaturing acrylamide gel electrophoresis. As indicated in Fig. 3B, both in vitro translated DNA MeTase and purified recombinant DNA MeTase form identical DNA-protein complexes.

**AdoMet Is Not Required for Generating a Stable Complex of DNA MeTase with the Substrate**—Previous studies using fluoro-substituted oligonucleotides have shown that covalent binding of DNA MeTase to the substrate occurs only in the presence of the methyl donor AdoMet (24). Our binding assays are performed in absence of AdoMet. As demonstrated in Fig. 3C, a DNA MeTase complex is formed whether or not AdoMet is present in the binding reaction mixture. This observation is consistent with the hypothesis suggested above that DNA MeTase forms a stable but not a covalent complex with the hairpin oligonucleotides. Alternatively, it is also possible that the direct inhibitor forms a stable complex with the product of the methylation reaction resulting in the absence of turnover which would also appear as low enzyme activity when AdoMet is present.

**Specificity of DNA MeTase Hairpin Interaction**—To determine whether the stable complexes formed between DNA MeTase and the hemimethylated hairpin are specific, we determined whether these complexes could be competed out by a cold excess of either specific or nonspecific oligonucleotides. First, we preincubated an A549 nuclear extract with a 40-fold excess (100 μM) of a nonspecific phosphorothioate single-stranded oligonucleotide (Scr), the hemimethylated hairpin oligonucleotide 3018, or with no competitor. A biotinylated hemimethylated hairpin bound to avidin-coated magnetic beads was then added to the reaction mixture and the different fractions of bound and unbound proteins were separated as described above. As a control, avidin-coated magnetic beads that were not bound to the hemimethylated hairpin were also tested (beads only). The hairpin-bound and unbound fractions were subjected to a Western blot analysis and DNA MeTase was visualized with a DNA MeTase antibody. The results of such an experiment presented in Fig. 4A show that binding of DNA MeTase to the hemimethylated hairpin is not competed out by preincubation with a nonspecific phosphorothioate oligonucleotide as indicated by the fact that DNA MeTase is eluted only in the boiled fraction. However, the specific hemimethylated hairpin oligonucleotide (3018) efficiently competes out the binding of DNA MeTase to the biotinylated hemimethylated hairpin as evidenced by its elution in the supernatant fraction. These results support the hypothesis that the interaction of DNA MeTase and the hemimethylated hairpin is specific.

We extended our study of the specificity of DNA MeTase and hairpin interactions by determining the ability of the different classes of hairpins studied in Tables II and III to compete out the binding of biotinylated hemimethylated hairpin to in vitro translated human DNA MeTase. As observed in Fig. 4B, phosphorothioate-modified hemimethylated hairpins bearing either inosine (3018) or cytosine (3016) in the methyl acceptor site 1 effectively compete out the binding of DNA MeTase as evidenced by the presence of DNA MeTase exclusively in the supernatant fraction. The 2’-O-methylated and phosphorothioate-modified hemimethylated hairpin (3060), which does not inhibit DNA MeTase, does not bind the DNA MeTase, as evidenced by the presence of DNA MeTase exclusively in the bound fraction. Similarly, the nonphosphorothioate hemimethylated hairpin 3046, which is not an inhibitor of DNA MeTase (Table II), does not bind in vitro translated DNA MeTase. The nonmethylated hairpin 3093 which inhibits DNA MeTase at lower potency (Table III) is a partial competitor as indicated by the presence of DNA MeTase in both the bound and the supernatant fractions. The nonmethylated inosine-modified hairpin 3056 which does not inhibit DNA MeTase at the concentrations studied in Table III is a weaker competitor as evidenced by the large fraction of DNA MeTase that remains bound to the hemimethylated hairpin after preincubation with this oligonucleotide.

These results were further confirmed by determining the ability of the 32P-labeled hairpins to form a DNA-protein complex with purified human DNA MeTase as determined by an electrophoretic mobility shift assay (Fig. 4C). This assay measures DNA-protein interaction per se but not the stable complex formation that is disrupted only by boiling, which is measured by the assay described above. As evidenced in Fig. 3C, oligonucleotides that inhibit DNA MeTase (Table II) form a DNA-protein complex (3016, 3018). Oligonucleotides that do not inhibit DNA MeTase (Table II and III) do not form a DNA-protein complex (3061, 3048, 3006, 3060, and Scr). However, nonmethylated hairpin 3093 which is not a potent inhibitor of DNA MeTase (Table III) can still bind it (Fig. 4C and D). Binding of the nonmethylated hairpin competes out the binding of DNA
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Fig. 4. Specificity of interaction of DNA Methylase with hairpin inhibitors. A, A549 nuclear extract. Ten μg of nuclear extract prepared from A549 cells were preincubated with 100 μM nonspecific phosphorothioate-modified oligonucleotides (Scramble) and 100 μM of the hemimethylated hairpin 3018, or with no competitor (control). Avidin-coated magnetic beads were added to the mixture as described under “Experimental procedures.” The hairpin-bound and unbound (Scramble, 3018) were added to the mixture as described above (Fig. 4D). Following 5 min of preincubation at room temperature, avidin-cleotides. This is consistent with a slow \( k_{on} \) of the hemimethylated hairpin for DNA Methylase.

In summary, the mechanism of action of the phosphorothioate-modified hemimethylated hairpin is consistent with the guiding principles underlying our design of DNA Methylase inhibitors; a potent DNA Methylase inhibitor exhibits high affinity binding to the enzyme and forms a stable intermediate DNA Methylase complex but is at best a poor acceptor of a methyl group.

Ex Vivo Activity of Hairpin Inhibitors of DNA Methylase: Inhibition of DNA Methylase and Tumorigenesis—It has been suggested that DNA Methylase inhibitors might be used therapeutically as antitumor agents (33, 34). We have previously shown that inhibition of DNA Methylase by antisense oligonucleotides in living cells or in tumors in vivo inhibits tumor growth (11). A critical question is whether the hairpin inhibitors developed in this study can be delivered into the nucleus, the subcellular site of DNA Methylase, whether they can stably bind and inhibit DNA Methylase in living cells and whether they can inhibit the growth of cancer cell lines?

This data is consistent with the ability of mammalian DNA Methylase to bind and methylate nonmethylated DNA (32). Whereas hemimethylation increases the rate of methylation (32) and the ability of modified DNA hairpins to inhibit DNA Methylase (table III), the ability to discriminate de novo and maintenance activities cannot be explained just by the differential capacity to bind the substrate. Both, methylated and nonmethylated hairpins form similar specific and identical DNA-protein complexes with DNA Methylase as demonstrated by the competition assay shown in Fig. 4D. However, a difference is observed in the ability of DNA Methylase to form stable complexes with methylated and nonmethylated hairpins (which are disrupted only by boiling) as determined by the biotinylated hairpin assay described above (Fig. 4B, 3056, 3093 versus 3018 and 3016).

Correlation of the Potency of Binding DNA Methylase, Inhibition of Enzymatic Activity, and Forming of a Stable DNA Methylase Complex by Hairpin Inhibitors—To test the hypothesis that the potency of inhibition of DNA Methylase by hemimethylated hairpins is dependent on its binding affinity to DNA Methylase, we compared the apparent \( K_d \) of hairpin 3016 for binding purified DNA Methylase as determined by a electrophoretic mobility shift assay (Fig. 5A, graphed in Fig. 6) and the apparent \( K_d \) for inhibiting DNA Methylase activity (Figs. 6 and 1). Both assays showed similar dose dependence (Fig. 6). The \( K_d \) for binding and \( K_d \) for inhibition of enzymatic activity are around 60–80 nM (Fig. 6). To further test the hypothesis that the hemimethylated hairpin forms a stable complex with DNA Methylase, we first bound purified recombinant DNA Methylase with \( 32^P \)-labeled hemimethylated hairpin (3018) and then challenged it with a thousand fold excess of cold hemimethylated hairpin. As observed in Fig. 5B, the \( 32^P \)-labeled hemimethylated hairpin oligonucleotide remains bound to the DNA Methylase up to 90 min after challenging with cold oligonucleotides. This is consistent with a slow \( k_{off} \) of the hemimethylated hairpin for DNA Methylase.

determined by an electrophoretic mobility shift assay. Purified human DNA Methylase was preincubated for 20 min with either 10 μM non-specific phosphorothioate oligonucleotide (Scramble) or with the hairpins as indicated in the figure (Competitor oligo) at room temperature. 1 μM of \( 32^P \)-labeled hemimethylated (3016) or nonmethylated (3093) hairpins were then added to the reaction mixture, the complexes were resolved by nondenaturing PAGE complexes corresponding to DNA Methylase (indicated by an arrow) were visualized by autoradiography.
Following a dose-response analysis of inhibition of DNA MeTase by fully methylated, hemimethylated, nonmethylated, and 2'-O-methyl-modified hairpin analogs as shown in Fig. 1, we have selected an active hemimethylated phosphorothioate hairpin (3016, $K_i = 65$ nM) and an inactive hemimethylated phosphorothioate 2'-O-methyl-modified hairpin analog (3060, $K_i > 1$ μM) for ex vivo studies. Both, the control and active hairpins are hemimethylated and phosphorothioate modified but differ in a modification of the sugar moiety. Non-small cell lung carcinoma A549 cells were treated with the active and control hairpins at concentrations of 10–100 nM in the presence of the lipid carrier, Lipofectin (Life Technologies, Inc.) (Fig. 7). To determine whether the active and inactive hairpins are delivered into the nucleus, the respective hairpins were tagged with fluorescein and the localization of the labeled oligonucleotide was followed by live cell fluorescence microscopy. As shown in Fig. 7C both the control and the active hairpins are localized to the nucleus as early as 1 h after treatment and remain in the nucleus up to 72 h post-transfection (data not shown).

Previous studies have shown that expression of an antisense mRNA to DNA MeTase or DNA MeTase antisense oligonucleotides inhibit the growth of murine adrenocortical carcinoma cells in vitro (11, 35). We determined whether the direct inhibitors of DNA MeTase developed in this study might have a similar effect on cell growth. An equal number of A549 cells were plated and treated with increasing concentrations of the DNA MeTase inhibitor (3016) and inactive hairpin control (3060). The cells were photographed (Fig. 7A) and counted (Fig. 7B) 72 h after treatment. As observed in Fig. 7 (A and B), direct inhibitors of DNA MeTase slow the growth of A549 tumor cells ex vivo, but do not have a nonspecific toxic effect on the cells. The number of cells increases after 3 days even in the presence of high dose (100 nM) of the hairpin inhibitor (3016) but to a lesser extent than nontreated cells or cells treated with a control oligonucleotide (3060). No toxicity is observed following 3 days of treatment with direct inhibitors as judged by morphological examination (Fig. 7A) or trypan dye exclusion (100% of the cells are viable).

To determine whether the hairpin inhibitor stably inactivated DNA MeTase in the living cell we assayed the activity of DNA MeTase in nuclear extracts prepared from hairpin inhibitor (3016) and hairpin control (3060) treated cells 72 h post-treatment. This assay detects differences in DNA MeTase activity only if the inhibitor remains stably bound to the enzyme.
during the purification protocol. As we have shown in Fig. 3A that the active hairpin is not removed from DNA MeTase even by 1 M NaCl, we reasoned that if the inhibitor interacted with the enzyme in the cell by a similar mechanism, it should remain bound to it even after salt extraction of nuclear extracts. As observed in Fig. 8A, there is a dose-dependent inhibition in DNA MeTase activity in nuclear extract prepared from active hairpin (3016)-treated cells but not in nuclear extracts prepared from control hairpin (3060)-treated cells.

To determine whether the reduction of DNA MeTase activity affected the tumorigenic potential of hairpin-treated cells we plated an equal number of active hairpin and control hairpin-treated cells in soft agar and onto regular tissue culture plates in parallel. Moreover, the treated cells could form colonies when plated on regular plastic dishes suggesting that the inhibitors are not generally toxic. To ensure that the reduction of soft agar colonies reflects a reversal of transformation rather than a toxic effect, the number of colonies on soft agar was divided by the number of colonies formed under regular tissue culture conditions. As observed in Fig. 8B, treatment with direct inhibitors of DNA MeTase results in a dose-dependent inhibition of anchorage independent growth that roughly parallels the effect observed on DNA MeTase activity shown in Fig. 8A.

A Western blot and quantification of the signal established that cells treated with 60 nM of the active hairpin bear a similar level of DNA MeTase protein to the nontreated control (Fig. 9B) which is consistent with the hypothesis that the reduction in MeTase activity observed in extracts prepared from hairpin-treated cells is a consequence of stable binding of the inhibitor to the enzyme.

Common wisdom suggests that inhibitors of DNA MeTase should cause demethylation of genomic DNA. In contrast, a comparison of the genomic level of methylation of CpG dinucleotides in cells treated with oligo 3016 and the control oligo 3060 using a nearest neighbor analysis has revealed no significant differences (data not shown). However, the results demonstrated in Fig. 9A show a limited demethylation of two HpaII sites located in the third exon of the retinoblastoma gene. As indicated in Fig. 9A, an expected 1-kilobase HpaII fragment appears after treating the cells with 10 nM hairpin 3016 and a second ~0.5-kilobase fragment is observed in cells treated with 60 nM of the active hairpin 3016 but not in cells treated with the control hairpin 3060.

To further test the hypothesis that DNA MeTase inhibitor treatment results in alterations in the DNA methylation pattern, we analyzed the state of methylation of a CG-rich exon of hairpin oligonucleotides. Phase contrast microscopy at × 100 magnification of A459 cells treated with a hairpin inhibitor of DNA MeTase and a control hairpin for 72 h. An equal number of A459 cells (1 × 10^5 cells per/cm plate) were treated with increasing concentrations (100 nM) of either hemimethylated hairpin 3016 or the 2'-O-methyl-modified hemimethylated 3060 hairpin control using Lipofectin as a lipid carrier three times every 24 h as described under "Experimental Procedures." B, cell number. A549 cells were treated with increasing concentrations of either hemimethylated hairpin 3016 or control hairpin 3060 as described above were harvested 72 h post-initiation of treatment and counted. The first bar indicates the number of cells at time 0. The following bars represent the number of cells after 3 days of treatment with either oligonucleotide. C, nuclear localization of hemimethylated and control hairpins. A549 cells were treated with a 5′ fluorescein-tagged hemimethylated hairpin (3118, identical to 3016) or the control hairpin (3188 identical to 3060) and the oligonucleotide was visualized by a fluorescence microscope 1 h after adding the oligonucleotide. The fluorescence micrographs are presented at a magnification of × 400. Whereas most of the fluorescence is localized to the nucleus, some fluorescence is diffusely present in the cytosol. Nontransfected cells show as expected no fluorescence (data not shown).
MyoD using bisulfite mapping. A summary of such an analysis presented in Fig. 9C shows that out of 41 CG sites in the region of MyoD analyzed 11 sites are less methylated, 10 sites are more methylated, while the remainder are unchanged. These results demonstrate that a reduction of DNA MeTase activity in the nuclei by direct inhibitors of DNA MeTase does not result in a broad inhibition of DNA methylation. Instead, the local changes in methylation at the specific sites observed indicate that the DNA methylation pattern is destabilized. We have similarly observed that in murine cells treated with a DNA MeTase antisense oligonucleotide hypomethylation of some sites was accompanied by hypermethylation of other sites.

DISCUSSION

A large number of DNA-binding proteins have been shown to play important physiological and pathophysiological roles. What makes these proteins excellent drug target candidates is the fact that their ligand is a specific DNA sequence. Therefore, a short double-stranded oligonucleotide bearing the protein recognition sequence could compete with the cognate sequence in the genome for binding to the protein and serve as a bona fide antagonist.

In addition to their specificity, the utilization of short DNA sequences as antagonists is attractive because of the relative ease by which these oligonucleotides can now be synthesized (1). Moreover, an already growing body of in vivo and clinical data provides an extensive base of information on the pharmacokinetics of oligonucleotides as well as their general nonspecific toxicity. However, a potential disadvantage of the use of competitive inhibitors of DNA-binding proteins, especially in a clinical situation, is the requirement for the accumulation of large quantities of the oligonucleotide inhibitor in the target cell (3, 36).

In this article we have demonstrated that this drawback can be mitigated by considering the recognition sequences of DNA-binding proteins as ligands of receptors. The classical pharmacological approach to the design of potent and efficacious antagonists of receptors has been to introduce limited chemical modifications to the basic ligand structure. The large number of modifications that can be added to the base sequence or phosphate-sugar backbone of oligonucleotides allows this same concept to be applied to the design of DNA-binding protein antagonists.

Using DNA MeTase as a model DNA-binding protein, this study demonstrates that like traditional receptor antagonists, the modification of oligonucleotides can significantly increase their specificity and antagonistic activity. In addition to identifying a number of lead DNA MeTase antagonists (EC50 of 30–60 nM) we would like to suggest that in the process of determining their potency of inhibition and the strength of their interaction with DNA MeTase we have been able to identify three distinct stages in the interaction of DNA MeTase and its substrate. The first essential stage involves the binding of DNA MeTase to its substrate, which does not significantly differentiate between nonmethylated and hemimethylated substrates. The next stage involves formation of a stable complex, which does not require AdoMet (Fig. 3C), but discriminates between hemimethylated and nonmethylated substrates. The next stage involves formation of a stable complex, which does not require AdoMet (Fig. 3C), but discriminates between hemimethylated and nonmethylated substrates as well as the base composition of the methyl acceptor site (Fig. 4B). This is followed by a covalent complex, which requires the presence of AdoMet resulting in methyl transfer and followed by the release of the DNA enzyme complex (15, 24, 32). Thus a modified oligonucleotide that binds the DNA substrate with high affinity, but in distinction from the cognate substrate is

A. D. Slack, N. Cervoni, M. Pinard, and M. Szyf, unpublished observations.
not an acceptor of a methyl group, will remain bound to the enzyme and serve as a potent and efficacious antagonist of DNA MeTase.

DNA MeTase has been recently proposed to be an important therapeutic target in cancer as well as other conditions (33, 34). This report has shown that DNA MeTase antagonists can be delivered into cells, inhibit DNA MeTase activity in human cancer cells, slow cellular growth, and inhibit anchorage independent growth, which is an indicator of tumorigenicity. A number of lines of evidence demonstrate that these effects are due to a specific inhibition of DNA MeTase. First, the hairpin oligonucleotides are potent inhibitors of DNA MeTase at the nanomolar range whereas other phosphorothioate oligonucleotides are not active even at the micromolar range. Second, there is a good correlation between the potency of the oligonucleotides and their specific binding to DNA MeTase. Third, the amount of the inhibitor (3016) required to reduce the amount of DNA MeTase activity that can be extracted from treated cells is similar to the amount of the inhibitor used in the in vitro methylation reactions (nanomolar range). Fourth, the potent inhibitors do not inhibit other activities such as DNA synthesis in a nuclear extract or HpaII MeTase. Fifth, there is a similar dose response for inhibition of DNA MeTase, cell growth, and anchorage independent growth. Sixth, the inactive analog (3060) has no effect on cell growth and anchorage independent growth. Seventh, whereas the inhibitor of DNA MeTase slows cell growth it has no nonspecific toxic effects. Eighth, whereas the inhibitor is localized to the nucleus 1 h after transfection, inhibition of DNA replication is only observed after 24 h, suggesting that the oligonucleotides do not have a nonspecific

**FIG. 9.** Effects of hairpin inhibitors on DNA methylation. A, analysis of methylation state of HpaII sites located in the Rb locus. DNA (10 μg) isolated from A549 cells treated with either increasing concentrations of hemimethylated hairpin (3016) or control hairpin (3060) were subjected to HindIII digestion followed by either HpaII (H) (which cleaves the sequence CCGG when the internal C is not methylated) or MspI (M) (which cleaves the sequence CCGG even when the internal C is methylated, digestion, 1.5% agarose gel fractionation, Southern blotting onto a Hybond N+ membrane, and hybridization with a 32P-labeled 1.4-kilobase HindIII fragment from plasmid pEB-8 (ATCC 57450). The position of the probe relative to the physical map of the Rb gene is underlined. The positions of the HindIII fragment and the expected MspI/HpaII fragments are indicated. B, Western blot analysis of DNA MeTase protein extracted from hairpin-treated cells. 50 μg of nuclear extracts prepared from A459 cells treated with Lipofectin alone, 60 nm hemimethylated hairpin 3016, or control hairpin 3060 were subjected to a Western blot analysis. DNA MeTase is visualized using a 1:2000 dilution of anti-DNA MeTase antibody (24) and an enhanced chemiluminescence detection kit (Amersham). Two bands are visualized. The signal obtained for the hemimethylated treated hairpin was similar to the signal obtained for Lipofectin treated controls. The amount of signal corresponding to DNA MeTase (OD arbitrary units) was normalized to the level of total protein transferred onto the membrane as determined by Amido Black staining and quantified by scanning (OD arbitrary units). The values obtained (OD of DNA MeTase signal divided by OD of the total protein staining): upper band: Lipofectin alone, 0.81; control hairpin 3060, 0.82; hemimethylated hairpin 3016, 0.80; lower band: Lipofectin alone 0.46; control hairpin 3060, 0.76; hemimethylated hairpin 3016, 0.56. C, bisulfite analysis of the first exon of the MyoD gene. DNA prepared from either A459 cells treated with the hairpin inhibitor (3016) or a control inhibitor were subjected to bisulfite treatment and the genomic region bearing 41 CpG sites in the first exon of the MyoD gene was amplified by polymerase chain reaction using the primers indicated under “Experimental Procedures” and sequenced. Eight clones were sequenced per DNA sample. The first line indicates the different CpG sites in the MyoD fragment. The percentage of methylated cytosines per site in the 8 clones analyzed per treatment were determined and are presented as different shadings of the circles representing each of the sites as indicated.
immediate effect such as inhibiting directly DNA polymerases (data not shown).

Whereas the common wisdom suggests that inhibitors of DNA MeTase should cause demethylation of genomic DNA, the results reported in this article report very limited demethylation induced by our inhibitors (Fig. 9C). However, since the hairpins described here do not actively remove methyl groups from DNA, they are expected to cause genome wide demethylation only if DNA replication proceeds at a high rate. As the hairpins described here effectively inhibited cell growth we did not expect wide demethylation to be induced by these inhibitors. Instead these results suggest that DNA methylation patterns reflect a dynamic balance of DNA MeTase activity, properties of the sequence and protein interactions at specific sites (34). Reduced methylation of some sites might disturb this balance, resulting in increased affinity of the DNA MeTase to other sites.

Certainly one of the future utilities of these inhibitors will be to determine the mechanism by which an inhibition of DNA MeTase slows cell growth. Two possible explanations for the antitumorigenic effects observed are: an active DNA MeTase is an essential component of the replication fork or, alternatively, the specific demethylation of some sites may trigger the activation of tumor suppressor genes. Regardless, this is the first documented example of direct inhibitors of DNA MeTase inhibiting DNA MeTase in living cells and they have helped to define the DNA-binding site of the DNA MeTase. Furthermore, the effectiveness of the inhibitors described here validate the use of modified oligonucleotides as bona fide antagonists of critical DNA-binding proteins.

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