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Inhibition of *Hha*I DNA (Cytosine-C5) Methyltransferase by Oligodeoxyribonucleotides Containing 5-Aza-2′-deoxycytidine: Examination of the Intertwined Roles of Co-factor, Target, Transition State Structure and Enzyme Conformation

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The presence of 5-azacytosine (ZCyt) residues in DNA leads to potent inhibition of DNA (cytosine-C5) methyltranferases (C5-MTases) *in vivo* and *in vitro*. Enzymatic methylation of cytosine in mammalian DNA is an epigenetic modification that can alter gene activity and chromosomal stability, influencing both differentiation and tumorigenesis. Thus, it is important to understand the critical mechanistic determinants of ZCyt's inhibitory action. Although several DNA C5-MTases have been reported to undergo essentially irreversible binding to ZCyt in DNA, there is little agreement as to the role of AdoMet and/or methyl transfer in stabilizing enzyme interactions with ZCyt. Our results demonstrate that formation of stable complexes between *Hha* I methyltransferase (M.*Hha* I) and oligodeoxyribonucleotides containing ZCyt at the target position for methylation (ZCyt-ODNs) occurs in both the absence and presence of co-factors, AdoMet and AdoHcy. Both binary and ternary complexes survive SDS-PAGE under reducing conditions and take on a compact conformation that increases their electrophoretic mobility in comparison to free M.*Hha* I. Since methyl transfer can occur only in the presence of AdoMet, these results suggest (1) that the inhibitory capacity of ZCyt in DNA is based on its ability to induce a stable, tightly closed conformation of M.*Hha* I that prevents DNA and co-factor release and (2) that methylation of ZCyt in DNA is not required for inhibition of M.*Hha* I.

Keywords: DNA methyltransferase; methylation inhibitor; 5-azacytidine; gene activation; epigenetics

Introduction

Enzymatic methylation of DNA at carbon 5 of cytosine has been shown to play a role in the regulation of a number of cellular processes through its ability to alter protein–DNA interactions. Changes in methylation status of specific regions of the genome have been implicated in the regulation of imprinting, maintenance of chromosome stability and altered gene expression in tumorigenesis.[1–3] Although it is well documented that early alterations in the process of DNA methylation during development of tumors result in global hypomethylation of genomic DNA,[4–7] a paradoxical hypermethylation of specific CpG islands has been linked to silencing of several genes involved in

Abbreviations used: Ade, adenine; AdoHcy, *S*-adenosyl homocysteine; AdoMet, *S*-adenosyl methionine; AP, abasic site; DNA C5-MTase, DNA (cytosine-C5)-methyltransferase; Cyt, cytosine; ds, double-stranded; DZCyt, 5,6-dihydro-5-azacytosine; FCyt, 5-fluorocytosine; MCyt, 5-methyl cytosine; M.*Eco* RII, *Eco* RII methyltransferase; M.*Hha* I, *Hha* I methyltransferase; M.*Hpa* II, *Hpa* II methyltransferase; MR Buffer, Methylation Reaction Buffer; ODN, oligodeoxyribonucleotide; ss, single-stranded; TE, Tris–EDTA; Ura, uracil; ZCyt, 5-azacytosine; ZCyd, 5-azacytidine; ZdCyd, 5-azadeoxycytidine.

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tumor suppression and mismatch repair.[8–10] In light of recent reports that some of these methylated genes can be reactivated by inhibitors of DNA methylation such as 5-azacytidine (ZCyd) and 5-aza-2′deoxycytidine (ZdCyd), and earlier studies showing that inhibition of methylation in certain tumor cell lines can induce differentiation[11,12] (and references in Zing *et al*.[13]), interest in the therapeutic uses of inhibitors of DNA methylation has been revived. ZCyd and ZdCyd have already been used as therapeutic agents for treatment of sickle cell anemia, myelodysplastic syndrome and several other cancers with varying degrees of success.[14–25]

In cells, ZCyd and ZdCyd are converted to deoxyribonucleoside triphosphates and subsequently incorporated into newly synthesized DNA in place of cytosine (Cyt) residues. DNA cytosine-C5 methyltransferase (C5-Mtase) is inactivated through binding to ZCyt residues that replace Cyt residues next to guanine (CpG), resulting in global hypomethylation of newly synthesized DNA.[11,26] Depending on the cell type, a variety of genes may be activated leading to differentiation or other alterations in phenotype. Unfortunately, ZCyd is cytotoxic, mutagenic and unstable in aqueous solution,[27–30] which limits its value for patient treatment. This has led us to investigate the potential of small, defined oligodeoxyribonucleotides (ODNs) containing ZCyt to serve as less toxic DNA C5-MTase inhibitors. Theoretically, such ODNs could act as specific inhibitors of DNA C5-MTases that do not require metabolic activation or incorporation into the host genome to inhibit DNA methylation. The studies described here were initiated to determine the critical mechanistic determinants of the inhibitory capacity of small ZCyt-ODNs. *Hha* I methyltransferase (M.*Hha* I), a well-characterized prokaryotic DNA C5-MTase was chosen as a surrogate for the larger, more complex mammalian enzymes because it shares a set of conserved catalytic domains with human and murine DNA C5 MTases. In addition, its interactions with small double-stranded ODNs and co-factors have already been defined by X-ray crystallographic studies.[31–37]

Previous reports describing the interactions of prokaryotic DNA C5-MTases, M.*Eco* RII,[38,39] M.*Msp* I, M.*Hha* I and M.*Hpa* II[39,40] with DNA or ODNs containing ZCyt residues provided support for a mechanism of inhibition involving formation of a covalent bond between the active site cysteine residue of the C5-MTase and C6 of ZCyt in DNA. However, the effect of co-factor on the formation of complexes containing DNA C5-MTases with DNA containing ZCyt in place of Cyt varied depending on the enzyme. Binding of M.*Eco* RII and M.*Hha* I was reported to occur in the absence of co-factor and to be stimulated in the presence of co-factor (AdoMet, AdoHcy, or Sinefungin)[38,39] while binding of M.*Msp* I and M.*Hpa* II were reported to be dependent on the presence of co-factor.[39] In separate reports, M.*Hpa* II and murine C5-MTase (DNMT1) binding to ZCyt-containing DNA were found to be equivalent in the presence or absence of AdoMet.[40,41] It remains to be determined whether the role of co-factor in formation of these complexes differs because of inherent differences in the enzymes, the substrates (biologically synthesized DNA with random substitution of ZCyt, or synthetic substrates with ZCyt in defined sites), or the method of assay. It is also unclear whether the transfer of methyl groups to ZCyt in DNA is a common feature of DNA C5-MTases. Early studies did not measure methylation directly, but it was inferred that methylation was not required for most DNA C5-MTases because complex formation occurred in the absence of methyl donor, AdoMet, and in the presence of AdoHcy, a competitive inhibitor of AdoMet binding.[26,39,42] However, there is one report of significant enzymatic methylation of ZCyt residues in DNA.[38]

The study reported here is unique in employing small double-stranded ODNs in which a ZCyt residue replaces the single target cytosine in a hemimethylated GCGC site (ZCyt-ODNs). We have assessed the effects of co-factor interactions on complex stability both by native gel electrophoresis and denaturing SDS-PAGE. Our results demonstrate that formation of extremely stable complexes between M.*Hha*I and ZCyt-ODNs occurs in both the presence and absence of cofactor and that M.*Hha*I in these complexes undergoes a dramatic conformational change. Co-factor (AdoHcy > AdoMet) significantly stabilizes M.*Hha*I/ZCyt-ODN complexes under non-denaturing conditions. However, cofactor has little effect on the thermal stability or conformation of complexes that are resistant to dissociation under denaturing conditions. Methylation of ZCyt residues during the formation of stable M.*Hha* I/ZCyt-ODN complexes in the presence of AdoMet could be detected but occurred with low frequency. The importance of these results to understanding the inhibitory mechanism of ZCyt residues in DNA is discussed.

Results

Inhibition of M.*Hha*I by ZCyt-ODNs

The small hemimethylated ODNs that were used here (Table 1) have already been shown to act as inhibitors of M.*Hha* I when 5-fluorocytosine (FCyt) is substituted for the target Cyt (underlined) in the M.*Hha* I recognition sequence 5′-G<u>C</u>GC-3′.[37] The relative inhibitory potency of ODNs containing ZCyt or FCyt in place of the target cytosine was compared to establish the effectiveness of ZCyt-ODNs as inhibitors of methylation by M.*Hha* I. Increasing concentrations of a 24 bp long substrate with a single hemimethylated 5′-GCGC-3′ site (AMp:A′, Table 1) were mixed with the indicated concentrations of inhibitor (15–120 nM). Methylation reactions were initiated by adding the

Table 1. Primary sequence of ss ODNs used in this study

ss ODN designation	Nucleotide sequence
BZ_7	5′ TGT CA**G** **Z**G**C** ATG G 3′
BX_7	5′ TGT CA**G** **X**G**C** ATG G 3′
$B'M_6$	5′ CCA T**GM** **GC**T GAC A 3′
A	5′ ATT **GCG** **C**AT TCC GGA TCC GCG ATC 3′
AFp	5′ ATT **GFG** **C**AT TCF GGA TCF GFG ATC 3′
A′Mp	5′ GAT MGM GGA TCM GGA AT**G** **MGC** AAT 3′

For simplicity, the following abbreviations are used in the text to refer to the ds ODNs formed by annealing these ODN pairs:ds ODNs designation, ss ODNs annealed; ZCyt-ODN, BZ_7 and $B'M_6$; AP-ODN, BX_7 and $B'M_6$; FCyt-ODN, AFp and A′Mp.

Sequence abbreviations: annealing of ss target strands (A, B) to complementary methylated ODNs (designated A′Mp, $B'M_6$) leads to the formation of ds ODNs that contain a single target base (underlined) in a hemi-methylated M.*Hha* I recognition site (boldface). Target base designations are abbreviated as Z, Zcyt; F, Fcyt; X, abasic furanose; M, MCyt. The position of the target base is indicated with numeric subscript. Fp or Mp indicates that all Cs in CpG sites in the ss-ODN have been replaced with FCyt or MCyt. The sequence of the B and B′ ODNs is the same as the ODNs used in M.*Hha* I crystal structure studies.[34,35,54]

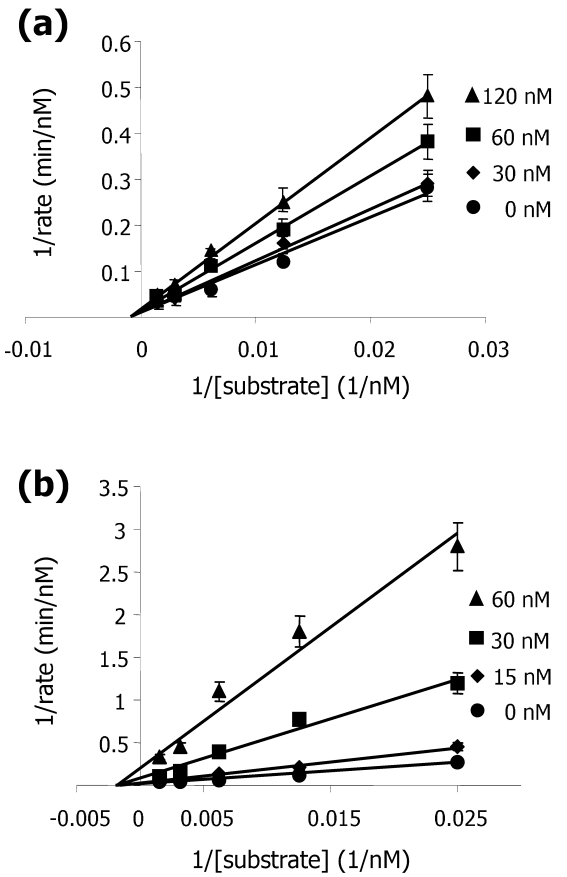

Figure 1. Inhibition of M.*Hha* I by FCyt- or ZCyt-ODNs. Methylation reactions containing 8.6 nM M.*Hha* I, 2.4 μM [^{3}H]CH_3-AdoMet, and the indicated concentrations of substrate (40–640 nM) were incubated (a) without inhibitor (●) or with 30 nM (◆), 60 nM (■), or 120 nM (▲) FCyt-ODN. In (b), the same concentrations of enzyme, cofactor, and substrate were incubated without inhibitor (●) or with 15 nM (◆), 30 nM (■), or 60 nM (▲) of ZCyt-ODN. AMp:A′ contains a single, hemi-methylated M.*Hha* I recognition site and target cytosine (see Table 1 for structures of FCyt and ZCyt). The rate of incorporation of [^{3}H]CH_3 groups was determined as described in Materials and Methods, and the inverse of the rate of substrate methylation was plotted *versus* the inverse concentration of substrate. Error bars indicate SD from the mean of three determinations. Absence of error bars indicates SD too small to illustrate.

substrate inhibitor mix to a standard reaction mixture containing 8.6 nM M.*Hha* I and 2.43 μM AdoMet. The rate of incorporation of radiolabeled methyl groups from [*methyl*-^{3}H]AdoMet was determined in a five minute reaction (Materials and Methods). It can be seen (Figure 1(a) and (b)), that both sets of data intersect at the *X*-axis in a Lineweaver–Burk plot, indicating non-competitive inhibition. This result is consistent with either covalent bond formation or high affinity non-covalent binding between M.*Hha* I and the inhibitor ODNs. The apparent K_i values derived from these data were 200(±73) nM for FCyt-ODNs (AFp:A′Mp) and 4.3(±0.65) nM for ZCyt-ODNs (BZ_7:$B'M_6$), indicating stochiometric formation of inhibitory complexes between ZCyt-ODNs and M.*Hha* I. While these K_i values suggest that ZCyt-ODNs are at least 50-fold more potent as inhibitors than FCyt-ODNs, such calculations are only accurate if all components of a reaction are in equilibrium. Since equilibrium cannot be reached when the inhibitor is covalently bound to the enzyme or when the rate of dissociation of the inhibitor from the enzyme is essentially zero during the reaction period, the K_i was determined by incubating 0.4 μM M.*Hha* I with increasing amounts of ZCyt- or FCyt-ODN for 0–5 minutes prior to addition of substrate. Using this method, the K_i for ZCyt-ODN was 0.7(±0.07) μM with a k_{inact} of 1.6(±0.06) min^{-1} compared to a K_i for FCyt-ODN of 1.0 (±0.09) with a k_{inact} of 0.78(±0.07) min^{-1}. Since the K_i values of ZCyt-ODN and FCyt-ODN are essentially the same and the rate of inactivation of M.*Hha* I is only ~twofold faster with ZCyt-ODN than with FCyt-ODN, these results indicate that an additional factor, the affinity of binding the inhibitory ODN relative to that of the normal substrate, accounts for the much higher apparent inhibitory capacity of ZCyt-ODN when added to enzyme concurrently with an excess of substrate (Figure 1).

Formation and dissociation of M.*Hha* I/ZCyt-ODN complexes

Native gel shift assays were used to evaluate the influence of cofactors on the interaction between M.*Hha* I and ZCyt-ODN and to test the previously reported findings that DNA C5-MTases bind very tightly (or are covalently linked) to ZCyt residues in DNA.[38,40–42] ZCyt-ODN (BZ_7:$B'M_6$, with ^{32}P-

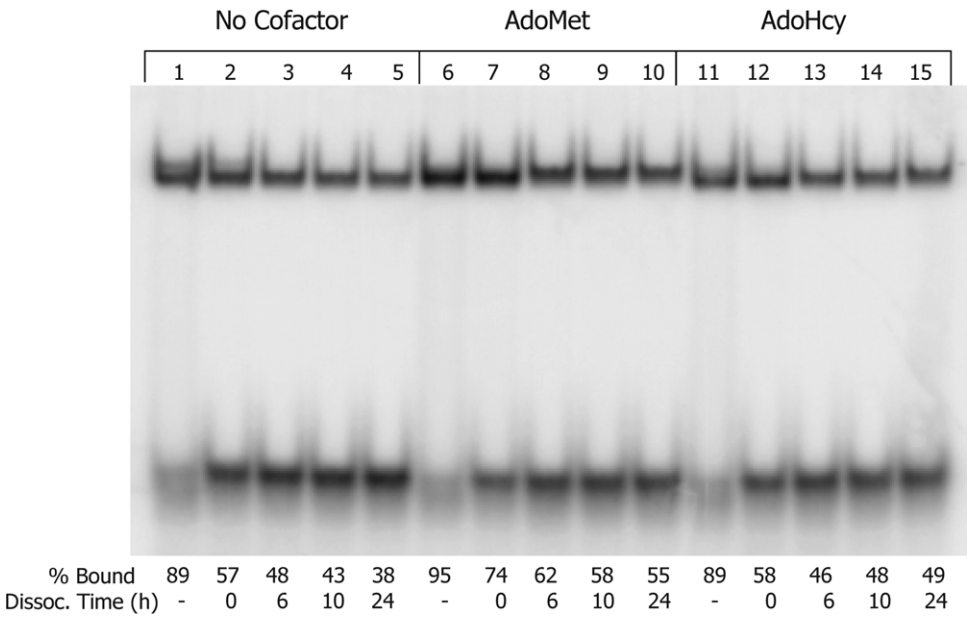

Figure 2. Effect of co-factor on M.*Hha*I/ZCyt-ODN complex dissociation. Binding reactions containing 45 nM ^{32}P-end-labeled ZCyt-ODN (BZ_7:B′M_6, with all radiolabel at the 5′ end of the B′M_6 strand), 108 nM M.*Hha*I, 75 ng poly-(dAdT:dAdT) either without co-factor (lanes 1–5) or with 100 μM AdoMet (lanes 6–10), or 100 μM AdoHcy (lanes 11–15) were incubated at 22 °C for 30 minutes. 100-fold excess (4.5 μM) of unlabeled ZCyt-ODN was then added to all binding reactions except those loaded in lanes 1, 6, and 11. Incubation was continued for times indicated. For 0 hour points, 100-fold excess of unlabeled ZCyt-ODN was added to the binding reaction mixture immediately before loading on a native polyacrylamide gel. Values for %Bound were derived from PhosphorImager analysis of the dried gel (%Bound = [counts in complex band/(counts in complex band + counts in free ODN band)] × 100).

radiolabel at the 5′ end of B′M_6) was incubated with M.*Hha*I in the presence or absence of co-factors for 30 minutes at 22 °C to allow formation of complexes. To determine whether the rate of dissociation of these binary and ternary complexes differed, 100-fold molar excess of unlabeled ZCyt-ODN was added to the reaction mixtures after initial complex formation, and the incubation was continued for up to 24 hours as indicated. Complexes were then separated from unbound ZCyt-ODNs by electrophoresis on non-denaturing 10% polyacrylamide gels (details are given in Materials and Methods and the legend to Figure 2, where the results of a typical experiment are presented). It can be seen that the presence of AdoMet in the reaction mixture increased the amount of M.*Hha*I/ZCyt-ODN complex formed (%Bound) compared to reaction mixtures containing AdoHcy or without co-factor (Figure 2, compare lane 6 to lane 1 or 11). This small but reproducible increase was observed in six separate experiments. However, in all the reactions more than 85% of the input radiolabeled ZCyt-ODNs were bound in binary (−co-factor) or ternary complexes (+AdoHcy, +AdoMet) in the presence of non-specific competitor DNA (poly(dAdT:dTdA)) (lanes 1, 6, and 11). Addition of specific competitor (100 fold-excess unlabeled ZCyt-ODN) caused an immediate decrease in the amount of bound radiolabeled ODN. The extent of this decrease was greatest for complexes formed with AdoHcy or in the absence of co-factor (32%, for lanes 1 and 2 and 31% for lanes 11 and 12). In the presence of AdoMet, approximately 20% of bound radiolabeled ODN was displaced (lanes 6 and 7). The primary source of these displaced ODNs was a "smear" of complexes that were present in all binding reactions that migrated more rapidly than the major band of complexes. A biphasic pattern of dissociation was observed for the remaining radiolabeled complexes. During the first phase, the rate of dissociation of both binary and ternary complexes was approximately the same, i.e. a plot of dissociation data from phase I (Figure 3 and Table 2) predicts a $t_{1/2}$ of 1–1.4 hours for all complexes. However, during phase II (dissociation from 2–30 hours) the effect of co-factor on dissociation becomes obvious (Figure 3 and Table 2). The rank order for co-factor stabilization of M.*Hha*I/ZCyt-ODN complex was AdoHcy > AdoMet > no co-factor. The data from phase II indicated that complexes without co-factor dissociated with $t_{1/2}$ values of approximately 4.5 days while the estimated $t_{1/2}$ for complexes containing co-factor was anywhere from 13 days (AdoMet) to >three weeks (AdoHcy). The results for binary complexes and ternary complexes with AdoMet are similar to those reported by Santi *et al.*,[40] who found no significant decrease in the amount of M.*Hpa*II/ZCyt DNA complexes formed in the presence or absence of AdoMet during three days incubation in the presence of 30-fold unlabeled competitor. Thus,

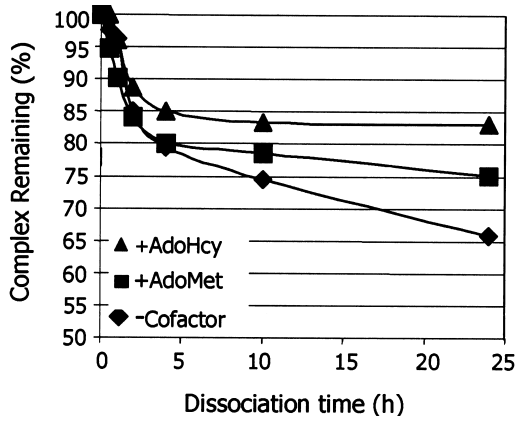

Figure 3. Analysis of M.*Hha* I/[^{32}P]ZCyt-ODN complex dissociation. The percentage of total ODN associated with M.*Hha* I was quantified as described in the legend to Figure 2 and plotted *versus* the duration of competition between unlabeled ZCyt-ODN and [^{32}P]ZCyt-ODN (BZ_7:^{32}P-B′M_6) complexed with M.*Hha* I. The presence or absence of cofactor during complex formation and dissociation is indicated (AdoHcy (▲), AdoMet (■), no cofactor (◆)). This analysis illustrates the rapid dissociation that occurs during the initial two hours of competition (phase I) compared with 100-fold slower dissociation between two and 24 hours (phase II).

Table 2. Summary of M.*Hha* I/ODN complex dissociation experiments

	Zcyt-ODN				
	Phase I		Phase II		AP-ODN
Co-factor present	k_{off} (hours^{-1})	$t_{1/2}$ (hours)	k_{off} (hours^{-1})	$t_{1/2}$ (hours)	$t_{1/2}$ (hours)
None	0.878	1.1	0.0093	110	90
AdoHcy	0.724	1.4	0.0009	1100	260
AdoMet	0.899	1.1	0.0032	310	180

The natural log of the percentage of total ODN associated with M.*Hha* I during native complex dissociation analysis (Figures 2 and 3) was calculated and plotted *versus* the duration of competition of pre-formed complexes containing radiolabeled ODNs with homologous, unlabeled ODNs. k_{off} (= −(slope) of the plot), and the $t_{1/2}$ (= 0.693/k_{off}) were determined from these plots. The dissociation of M.*Hha* I complexes containing ZCyt-ODN in the absence of co-factor, or presence of AdoHcy or AdoMet is presented as two sets of resulting k_{off} and $t_{1/2}$ values for phase I (0–2 hours competition) and phase II (2–24 hours competition) because of the observed biphasic dissociation of the complexes (Figure 3). A single set of k_{off} and $t_{1/2}$ values is presented for dissociation of AP-ODN in the presence or absence of cofactors since the dissociation plot was linear.

extremely stable complexes between M.*Hha* I and ZCyt in DNA are indeed possible in the absence of AdoMet and their stability is greatest in the presence of the methylation inhibitor AdoHcy. This strongly supports the hypothesis that the mechanism of M.*Hha* I inhibition by ZCyt-containing DNA does not require methylation. Since it has been demonstrated that hydrolysis of ZCyd in solution leads to ring-open forms, which can undergo further degradation,[43] we compared the dissociation of M.*Hha* I/ZCyt-ODN and M.*Hha* I/AP-ODN (BX_7:B′M_6) complexes (AP = abasic furanose target) to determine the effect of complete

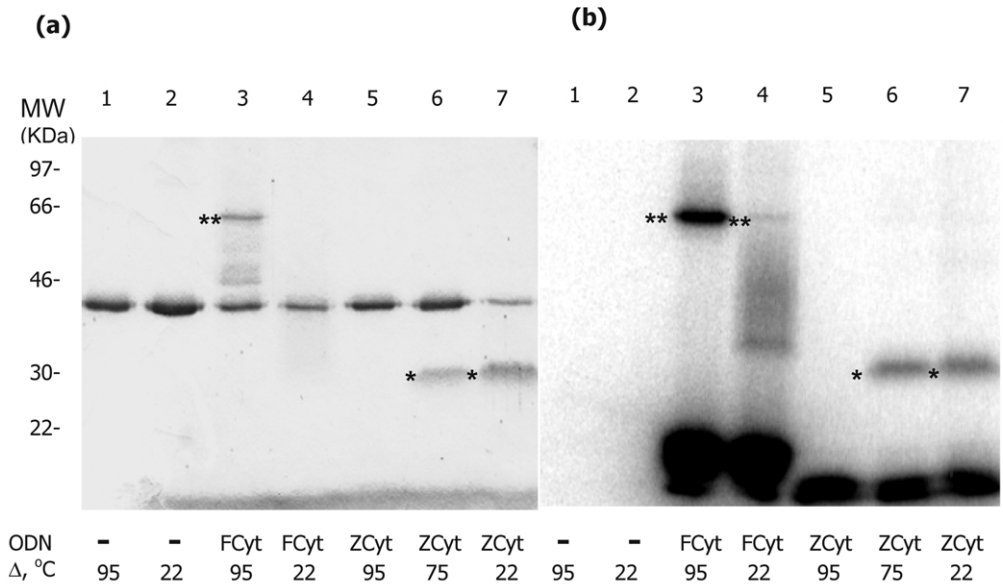

Figure 4. Analysis of M.*Hha* I complexes with FCyt and ZCyt-ODNs under denaturing conditions. Reactions containing 1.35 μM M.*Hha* I and 200 μM AdoMet (omitted from reactions in lanes 1 and 2) were incubated for one hour at 37 °C either in the absence of ODN (lanes 1 and 2) or with 2.96 μM FCyt-ODN (lanes 3 and 4) or ZCyt-ODN (lanes 5–7). The 5′end of the ODNs containing FCyt or ZCyt was ^{32}P-radiolabeled. Following complex formation, 1% SDS and β-mercaptoethanol were added. The samples were either held at room temperature (lanes 2, 4 and 7) or heated for five minutes at 95 °C (lanes 1, 3 and 5) or 75 °C (lane 6), loaded unto a 10% SDS/polyacrylamide gel, and electrophoresed at 200 V for ~60 minutes. The resulting gel was stained with Coomassie blue (a) and examined by autoradiographic imaging (b). The molecular weight markers are to the left of (a). (**) indicates the most slowly migrating form of FCyt/M.*Hha* I complex in both gel images. (*) indicates the ZCyt/M*Hha* I complex.

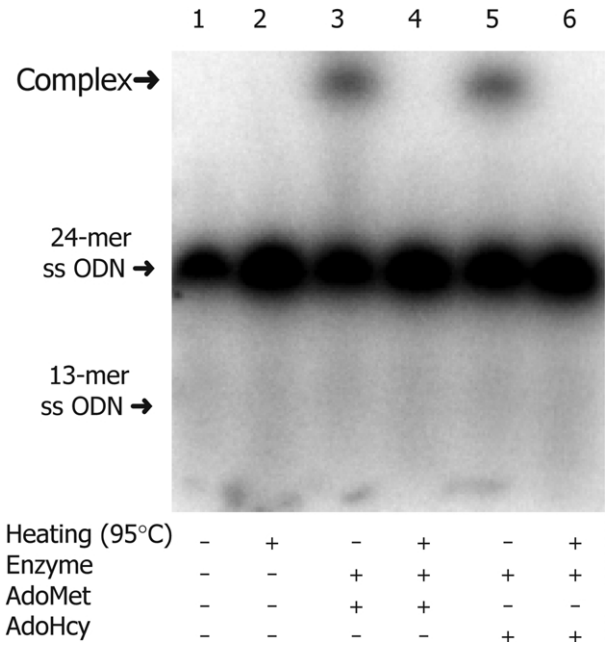

Figure 5. Effect of heating on the integrity of ZCyt-ODNs before and after formation of complexes with M.*Hha* I. Reactions contained 0.256 μM of a 24 bp long ^{32}P-5′-end-labeled ZCyt-ODN (5′-^{32}P-TGTCA**G ZGC**ATGGATGGTTATAAT-3′ and 5′-ATTATAACCAT-CCAT **GMG C**TG ACA-3′), 0.216 μM M.*Hha* I (lanes 3–6) and 0.16 mM AdoMet (lanes 3 and 4) or AdoHcy (lanes 5 and 6). After one hour incubation at 37 °C for 60 minutes to allow complex formation, reactions were brought to 1% SDS and β-mercaptoethanol, heated at 95 °C (+, lanes 2, 4 and 6) or 50° C (lanes 1, 3 and 5) for five minutes. Samples were analyzed on a 15% acrylamide/8 M urea gel. The arrows indicate the migration of ss 24-mer ODNs and 13-mer ODNs used as markers. The first 13 bases of the ^{32}P-radiolabeled strand and the last 13 of the unlabeled strand are identical to those in B:B′M$_6$. The additional bases were added to enhance separation between the intact radiolabeled strand and its potential scission products. This autoradiograph illustrates that even though complexes were destroyed by heating to 95 °C, no scission products were detected.

absence of a normal base on binding. Crystallographic studies have demonstrated that the deoxyribose in AP-ODN is flipped into the active site pocket in ternary complexes with M.*Hha* I and AdoHcy.[44] Our data indicate that surprisingly stable complexes between M.*Hha* I and AP-ODN also form in the absence or presence of co-factor (Table 2) but have a monophasic dissociation curve (data not shown). The distinct difference between the patterns and rates of dissociation of M.*Hha* I/AP-ODN and M.*Hha* I/ZCyt-ODN complexes indicates that neither the rapidly nor the slowly dissociating M.*Hha* I/ZCyt-ODN complexes have undergone complete loss of the ZCyt pyrimidine base. In phase II of dissociation, M.*Hha* I complexes formed with ZCyt-ODNs in the absence of co-factor have similar $t_{1/2}$ values to complexes formed with AP-ODNs. However AP-ODN complexes are much less sensitive to the stabilizing effect of co-factor. For example, AdoHcy increases the $t_{1/2}$ for M.*Hha* I/ZCyt-ODN complexes >tenfold while the $t_{1/2}$ for M.*Hha* I/AP-ODN complexes is only increased <threefold in the presence of AdoHcy. This suggests that the interaction between the base and co-factor in complexes is the primary influence on the stability of enzyme–DNA complexes.

Binding to ZCyt-ODN induces a stable conformational change in M.*Hha* I

In ternary complexes with FCyt-ODNs, the target FCyt is flipped out of the DNA helix,[34] methylated and covalently linked to M.*Hha* I. The covalent bond in FCyt-ODN ternary complexes is stable to heating at 95 °C in the presence of 1% SDS and 1% β-mercaptoethanol. M.*Hha* I/ZCyt-ODN complexes are completely dissociated under these conditions (Figure 4) even though there is no scission of the ZCyt-ODNs at 95 °C (Figure 5). To examine the stability of ternary complexes containing ZCyt-ODNs in greater depth, we compared FCyt and ZCyt-ODN ternary complexes using SDS-PAGE analysis. Radiolabeled ds-FCyt and ZCyt-ODNs were formed by annealing ^{32}P-radiolabeled AFp or BZ$_7$ with A′Mp or B′M$_6$ (Table 1) to allow simultaneous detection of both the ODN and M.*Hha* I. In the absence of ODN, the electrophoretic mobility of M.*Hha* I after SDS-PAGE was identical regardless of whether the enzyme was heated to 95 °C or maintained at 22 °C in the presence of SDS and β-mercaptoethanol prior to electrophoresis (Figure 4(a), Coomassie blue-stained gel, lanes 1 and 2). Incubation of M.*Hha* I with FCyt-ODN and AdoMet at 37 °C for one hour, followed by treatment with SDS and β-mercaptoethanol at 22 °C did not alter its migration from that of the free enzyme (Figure 4(a), lane 4) when examined by Coomassie blue staining. However, the amount of enzyme appeared to be less than that loaded (compare with Figure 4(a), lanes 1 and 2). As demonstrated by the electrophoretic behavior of M.*Hha* I complexes with bound [^{32}P]FCyt-ODN (Figure 4(b), lane 4), the apparent loss of protein can be accounted for by the widespread distribution of the complexes over the gel lane (apparent molecular mass 33–59 kDa). After heating to 95 °C, the intensity of Coomassie blue-stained free protein band was increased and a slower migrating band with the mobility predicted for a complex containing one molecule of FCyt-ODN (AFp:A′Mp) Mp) covalently bound to one molecule of M.*Hha* I (~59 kDa) appeared along with an array of protein bands of intermediate mobility (Figure 4(a), lane 3). Only the slowest migrating band contained [^{32}P]FCyt-ODN (Figure 4(b), lane 3). Thus, most of the bound enzyme had not formed covalent complexes with FCyt-ODN during the incubation period. In contrast, ternary complexes of M.*Hha* I with [^{32}P]ZCyt-ODN actually migrated more rapidly than free enzyme after treatment with 1%

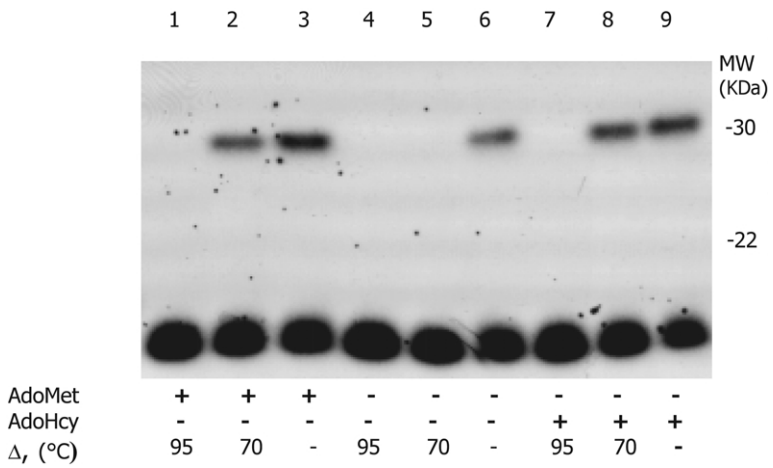

Figure 6. Analysis of the effect of co-factor on stability of M.*Hha* I/ZCyt-ODN complexes under denaturing conditions. Reactions containing 41 nM [^{32}P]ZCyt-ODN ([^{32}P]BZ_7:$B'M_6$), 108 nM M.*Hha* I, and 100 μM co-factor (as indicated) were incubated at 37 °C for 60 minutes. Reactions were terminated by bringing them to a final concentration of 10% glycerol, 1% SDS, and 1% 2-mercaptoethanol. Products were either left unheated or heated at indicated temperatures for five minutes prior to loading and electrophoresed as described in the legend to Figure 5. An autoradiographic image is shown with molecular weight markers indicated to the right.

SDS and β-mercaptoethanol at 22 °C (compare Figure 4(a) and (b), lane 7 with (a) lanes 1 and 2). As estimated from the intensity of Coomassie blue staining, approximately 70% of the input enzyme protein was present in a rapidly migrating complex with [^{32}P]ZCyt-ODNs (Figure 4(a) and (b), lane 7). This complex, which has an electrophoretic mobility consistent with a 30 kDa protein, persisted even after heating to 70 °C for five minutes prior to electrophoresis (Figure 4(a) and (b), lane 6). However, when an aliquot of the same reaction mixture was heated to 95 °C, all of the protein migrated with the mobility expected for completely denatured M.*Hha* I (Figure 4(a), compare lanes 5 and 1) and the ODN migrated with the mobility expected for the free ODN (Figure 4(b), lane 5). Thus, it is clear that the higher mobility complex is not simply the result of enzyme degradation during the reaction with ZCyt-ODNs.

These experiments indicate that complexes involving M.*Hha* I and ZCyt-ODNs that are stable in the presence of SDS and β-mercaptoethanol migrate with a higher mobility in denaturing SDS gels because of a reversible change in the conformation of M.*Hha* I that results from its interaction with the ZCyt target. The mobility of this complex differs markedly from that of the complex resulting from M.*Hha* I methylation of and covalent linkage to an FCyt-ODN. Without heating, only a small proportion of the ternary M.*Hha* I/[^{32}P]FCyt-ODN/AdoMet complexes migrate more rapidly than free enzyme (Figure 4(b), lane 4), while heating at 95 °C converts all of the covalently linked complex to a low mobility form that migrates at the rate expected for M.*Hha* I covalently linked to FCyt-ODN.

ZCyt-ODN induction of M.*Hha* I conformational change is independent of co-factor

To further characterize the novel conformation of M.*Hha* I complexed to ZCyt-ODN, we compared the stability of M.*Hha* I/ZCyt-ODN complexes formed in the presence of AdoMet, AdoHcy or in the absence of co-factor. Complexes maintained at 22 °C were found to have the same electrophoretic mobility in both native and denaturing gels regardless of whether co-factor was present or not (Figure 2 and Figure 6, lanes 3, 6 and 9). When heated to 70 °C prior to SDS-PAGE, complexes containing AdoMet or AdoHcy remained intact although the proportion of bound/free ZCyt-ODN in the reaction mixture was somewhat reduced. Under the same conditions, ZCyt-ODN was almost completely dissociated from complexes with enzyme alone (Figure 6, compare lanes 2 and 8 with lane 5). This result suggested that while co-factor is not a requirement for formation of high mobility M.*Hha* I/ZCyt-ODN complexes, the presence of co-factor does stabilize the complex against dissociation by heat in the presence of 1% SDS and β-mercaptoethanol. However, the rank order for amount of complex formed as detected in this assay was the same as that observed using native gel electrophoresis (AdoMet > AdoHcy > no co-factor). Thus, when the amount of complex remaining after heating to 50 and 75 °C was normalized to the amount of complex present after treatment with 1% SDS and β-mercaptoethanol at 22 °C (Figure 7), it became obvious that the effect of temperature on the rate of dissociation is similar whether co-factor is present or not, i.e. co-factor does not contribute significantly to heat stabilization of these compact, high mobility complexes.

Detection of methylation of ZCyt-ODNs by M.*Hha* I

While the studies on the effects of cofactor on M.*Hha* I/ZCyt complex stability presented above clearly demonstrate that methyl transfer is not necessary for complex formation, they do not rule out the possibility that methyl transfer can occur during ternary complex formation in the presence of AdoMet. X-ray crystallographic analysis provided the final proof that when AdoMet is present,

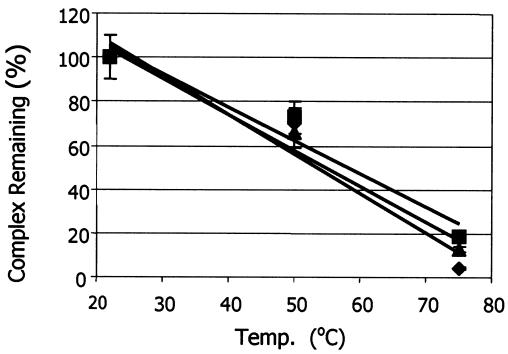

Figure 7. The effect of increasing incubation temperatures prior to SDS-PAGE analysis of M.*Hha* I/ZCyt-ODN complexes.To determine the effect of cofactor on complex stability under denaturing conditions, M.*Hha* I/[^{32}P]ZCyt-ODN complexes formed in the absence of cofactor (◆), or presence of excess AdoMet (■) or AdoHcy (▲) were either left unheated or heated at 50 or 75 °C prior to SDS-PAGE. All details are as described in the legend to Figure 6. The total percentage of SDS-resistant complex present in unheated reactions after SDS-PAGE was set as 100%. The percentage of complex remaining was plotted *versus* the increasing temperature of heating prior to loading. Error bars indicate SD from the mean of three determinations. Absence of error bars indicates SD too small to illustrate.

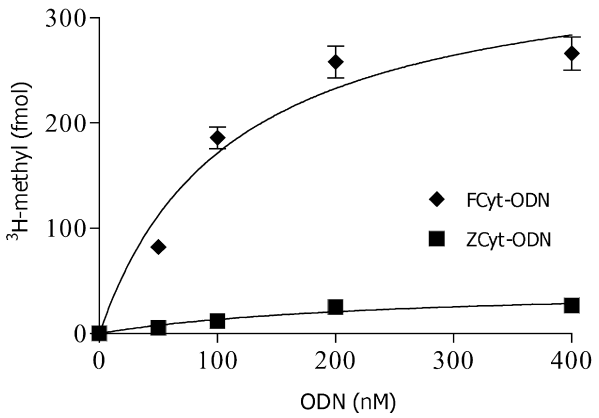

Figure 8. Comparison of FCyt- and ZCyt-ODN methylation by M.*Hha* I. Reaction mixtures containing 173 nM M.*Hha* I, 450 nM [^{3}H]CH_3-AdoMet (specific activity 84 Ci/mmol), and increasing concentrations (50 nM, 100 nM, 200 nM, or 400 nM) of ZCyt-ODNs (■) or FCyt-ODNs (◆) were incubated at 37 °C for 30 minutes. Reaction products were precipitated in perchloric acid as described[37] and the amount of methyl transfer to the ODNs was determined by liquid scintillation counting. Error bars indicate SD from the mean of three determinations. Absence of error bars indicates SD too small to illustrate.

M.*Hha* I forms a covalent complex with FCyt in DNA and that carbon 5 of the flipped target Cyt in the covalent complex carries both a fluorine and a methyl group.[34] Even though transfer of this methyl group results in the inactivation of the enzyme, it is possible to directly measure methyl transfer to FCyt-ODN using high concentrations of enzyme and [*methyl*-^{3}H]AdoMet with a specific activity of ~3000 GBq/mmol.[37] The ability of M.*Hha* I to catalyze methyl transfer to FCyt and ZCyt-ODN was compared (Figure 8). The maximal rate of methyl transfer per fmol M.*Hha* I protein was 0.002(±0.0002) fmol/minute to FCyt-ODN and 0.2(±0.03) × 10^{-3} fmol/minute to ZCyt-ODN. Since both substrates have a similar K_i, these results directly support the evidence provided by the binding assays described above, i.e. that the inhibitory interaction between ZCyt-ODN and M.*Hha* I does not require methylation. Although it is not possible to rule out the possibility that some loss of radiolabeled, methylated ZCyt-ODN occurred during collection of the ODN by acid precipitation, no significant scission of ZCyt-ODN occurred during a one hour incubation at 22 °C in the presence of 80% acetic acid[45] or during acid precipitation (data not shown). Furthermore, when *methyl*-^{3}H incorporation was determined using an alternative method carried out at neutral pH, binding of enzyme–ZCyt-DNA complexes to a nitrocellulose filter, followed by washing with 0.3 M NaCl and 0.1% Sarkosyl,[41] radiolabel in M.*Hha* I/ZCyt-ODN complexes was not significantly greater than background while the level of *methyl*-^{3}H in enzyme M.*Hha* I/FCyt-ODN complexes was comparable to that shown in Figure 8.

Discussion

In 1984, Santi proposed that methylation by DNA C5-MTases involves reversible formation of a dihydropyrimidine intermediate with a covalent linkage between the enzyme and C6 of the target Cyt in DNA and a carbanion equivalent at C5. This reactive intermediate could either (1) accept a proton from water or a general acid of the protein or (2) a methyl group from AdoMet. Abstraction of a proton from C5 followed by β-elimination of the enzyme nucleophile would lead to release of the DNA with either (1) an unmethylated or (2) a methylated cytosine.[40] It was also proposed that methylation of FCyt or addition of a proton to N5 of ZCyt would lead to formation of a stable covalent bond between enzyme and substrate (see scheme for ZCyt, Figure 9, structure IIb). Formation of C5-MTase/FCyt-DNA complexes stable to heating at 95 °C in the presence of SDS and a reducing agent has been reported by a number of investigators and the predicted methylated FCyt intermediate with M.*Hha* I has been demonstrated in X-ray crystallographic studies.[34] The occurrence of covalent complexes with ZCyt residues in DNA is also supported by evidence of essentially irreversible complexes between ZCyt-substituted DNA and C5-MTases from bacterial and mammalian cells *in vitro*,[38–41] and *in vivo*.[11,30,46] However, there is little agreement regarding the role of cofactors and/or methyl transfer in complex formation and there is as yet no X-ray crystallographic data on the structure of ZCyt complexes with M.*Hha* I. Thus, the findings reported here provide several unique insights into the mechanism of

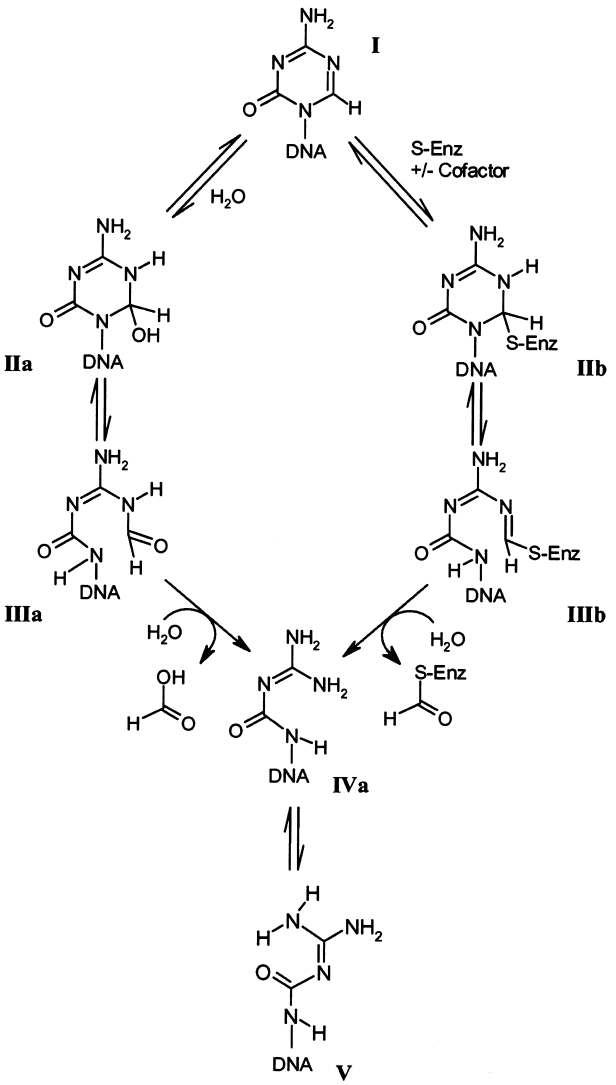

Figure 9. Predicted structural changes in ZCyt in DNA. (I) Intact Zcyt. (IIa) Dihydrocytosine intermediate form of ZCyt following addition of water across the C6═N5 double bond. (IIb) Dihydrocytosine intermediate form of ZCyt following enzymatic attack at C6; ring open form of ZCyt in the absence (IIIa) or presence (IIIb) of DNA C5-MTase. Following hydrolysis of either ring open form of ZCyt, the product would be form IVa, which could take on the conformation shown as form V. Theoretically, form V in DNA can direct incorporation of Cyt during DNA replication leading to C:G → G:C transversions.[30]

methyl transfer and the probable structure of the M.*Hha* I/ZCyt-ODN complex.

Formation and stabilization of ZCyt-ODN complexes

The data presented here suggest that methylation of ZCyt residues in DNA can occur, but that the probability of transfer of a methyl group by M.*Hha* I is much lower than the probability of transfer of a proton leading to covalent bond formation. A rigorous evaluation of the rate and extent of formation of 5-methyl-5-azacytosine in ODNs will require mass-spectrophotometric analysis. However, the evidence presented here indicating that methylation is not required for formation of stable complexes between ZCyt-ODN and M.*Hha* I is consistent with Santi's proposal that addition of a proton at N5 can lead to formation of a stable but slowly reversible covalent link between enzyme and ZCyt in DNA.

On the basis of X-ray crystallographic and NMR studies, it has been proposed that enzyme–DNA complexes with at least three different structures are involved in the mechanism of action of M.*Hha* I. These include (1) an initial complex formed between the enzyme and a normally stacked *B*-DNA helix, (2) an "open" complex of enzyme and DNA composed of "an ensemble of flipped out conformers", and (3) a more compact or closed complex in which the active site loop of the enzyme locks the flipped out target cytosine into the active site pocket.[47] The "closed" configuration is manifested by a more rapid migration of ternary complexes with AdoHcy than for binary complexes in native polyacrylamide gels.[37,48] Formation of closed complexes between M.*Hha* I and ODNs with Cyt or mismatched base (Ade, Ura) targets, as defined by native gel migration rates are only detectable in the presence of AdoHcy.[37,48] In contrast, ODNs containing 5,6-dihydro-5-azacytosine (DZCyt) or ZCyt targets form closed complexes with M.*Hha* I in both the presence and absence of co-factors (Figure 2 and Sheikhnejad *et al*.[37]). We postulate that a DZCyt target, which cannot be methylated or form a covalent complex, either induces or allows a shift of equilibrium toward the closed M.*Hha* I conformation in the absence of co-factor because the shape of DZCyt in the catalytic pocket so closely mimics the shape and non-aromatic character of a dihydrocytosine reaction intermediate.[37] This would be expected to reduce the energetic requirements for the massive conformational changes that occur when the target base is locked into the catalytic pocket, as it is when crystallized in the presence of AdoHcy.[48] If the non-aromatic character of the target base that occurs with covalent bond formation is not only sufficient but also necessary for formation of closed complexes in the absence of co-factor, the results presented here (Figures 2 and 6) support the hypothesis that formation of covalent complexes between M.*Hha* I and ZCyt in DNA (Figure 9, IIb) does not require co-factor. Our results indicate that co-factor can, however, affect the rate of formation and stability of the complexes. It is consistently observed that the presence of AdoMet increases the amount of closed complex formed by 20–30% relative to the amount of closed complex formed in the presence of AdoHcy or in the absence of co-factor (Figures 2 and 6). However, the primary effect of the S^{+}-CH3 moiety of AdoMet seems to be on rate of formation or initial stabilization of the complex rather than on its long-term

stability. AdoHcy increases the $t_{1/2}$ for dissociation ~3.5-fold more than AdoMet (Table 2).

Influence of ZCyt structure and breakdown on complex stability

There is some probability that at least three different forms of ZCyt are present in the ODNs used in our binding studies (Figure 9): intact ZCyt (structure I), the initial hydrolysis product of ZCyt which can be either in a non-aromatic ring or a ring open form (structures IIa, IIIa), and the ring open form that has undergone loss of the formyl group (structure IVa). However, mass spectrometric analysis revealed only small amounts of two alternate forms of ZCyt-ODN. One form had a loss of ten mass units, as would be predicted for an ODN with the ring open form following loss of the formyl group (structure IVa) and the other 290 mass units, the mass of a ZCyt nucleotide.[45,49] The ZCyt-ODNs used in these studies were purified by ion exchange chromatography and no longer contained detectable amounts of the single nucleotide deletion ODN (see Figure 5). Thus, it is most probable that the first phase of dissociation (Figure 3 and Table 2) involves complexes formed between M.*Hha* I and ODNs containing the pre-existing ring-open form IVa (ZCyt-IVa-ODN) in the target position. This proposal is supported by two aspects of the experimental data for phase I dissociation, namely its low $t_{1/2}$ and the relatively minor stabilization by cofactor. While the possibility exists that several of the structural features of the cytosine ring that interact with amino acids in the catalytic pocket of M.*Hha* I are maintained in ZCyt-IVa-ODN,[35] ZCyt-IVa-ODN has lost any possibility of stabilization through formation of a partial or complete covalent bond between C6 of ZCyt and Cys81 of M.*Hha* I. In addition, formation of an alternate ring structure through intramolecular hydrogen bond formation (structure V) could interfere with enzyme–DNA interactions such as the one between O2 and Arg165. Thus, it is probable that the flipped target base of ZCyt-IVa-ODN behaves similarly in its interactions with amino acids in the catalytic pocket to a non-cytosine (mismatched) base. This hypothesis is supported by the observation that the $t_{1/2}$ for dissociation of M.*Hha* I complexes with ODNs containing Ade and Ura targets is in the range of 15–90 minutes[37,48] similar to the $t_{1/2}$ of 60–80 minutes for ZCyt-ODNs in phase I.

Binary complexes between M.*Hha* I and ODNs with Ade or Ura targets are in the open conformation and are only stabilized in the closed conformation by AdoHcy, not by AdoMet.[37,48] Typically, the degree of stabilization observed with the shift from open to closed complex with these mismatched bases is at least fivefold less than that observed with ODNs having Cyt or Cyt analogs as the target base. The phase I ternary complexes with ZCyt-ODN and AdoHcy are stabilized by no more than 50% relative to phase I binary complexes while AdoHcy increases phase II complex stability by tenfold. Careful examination of the native gel shift assay shown in Figure 2 suggests that only a minor proportion of binary complexes (<10%) migrate with the slower rate characteristic of open complexes. These complexes dissociate within two hours of addition of cold competitor (Figures 2, 3 and data not shown), a result consistent with the predicted M.*Hha* I binding properties with ZCyt-IVa-ODN and the proposed role of AdoHcy in stabilizing a flipped base in the catalytic pocket through long-range electrostatic interactions with the base and the two flanking phosphate groups.[35]

The first clue as to the structure of ZCyt in the highly stable phase II complexes comes from their observed electrophoretic mobility in native gels (Figure 2). All of the complexes, regardless of the presence or absence of co-factor, migrate with the increased mobility that is postulated to be the result of the closed configuration assumed by M.*Hha* I when the active site loop (residues 80–90) locks the flipped-out target into the catalytic pocket.[35] While there is as yet no high resolution crystal structure of M.*Hha* I with ZCyt-ODN, comparison of the crystal structures of DZ13, a ternary complex of AdoHcy, M.*Hha* I and a hemimethylated substrate with a DZCyt target and HM13, a ternary complex of AdoHcy, M.*Hha* I and a hemimethylated ODN with a Cyt target indicated only a few major differences. These lie in the structure of the pyrimidine rings and the distance between sulfur of the Cys81 and C6 (3.1 Å in DZ13 *versus* 2.3 Å for the "partial" covalent bond distance between Cys81 and C6 in HM13; see O'Gara *et al*.[35] and Sheikhnejad *et al*.[37] for other details). Since Cyt does not form closed complexes in the absence of co-factor, but the transition state mimic of ZCyt (DZCyt) does, our results suggest that the base in the initial phase II complex has a similar structure to DZCyt. This would be a closed complex in which covalent bond formation has occurred leading to a non-aromatic ring structure (structure IIb). Our finding that the $t_{1/2}$ for dissociation of the more labile binary complex with ZCyt-ODN is on the order of days (Table 2) rather than hours, as was observed for non-covalent M.*Hha* I complexes formed with DZCyt-ODN ([37] and A.S.B. – J.K.C., unpublished results), supports this idea. Since M.*Hha* I/ZCyt-ODN/AdoHcy complexes do not exhibit significant dissociation over 24 hours, it seems likely that the preservation of all of the van der Waals and electrostatic contacts between AdoHcy and ZCyt-IIb, plus a potential hydrogen bond to NH_2 through water, stabilize the ZCyt residue in the transition state (IIb), preventing reversion to structure I or ring-open forms (structures IIIb or IVa). Modeling of AdoMet in the HM13 structure indicates that the methyl group of AdoMet greatly increases the electrostatic potential of the co-factor compared with AdoHcy, leading to the extension of AdoMet further into the DNA.[50] It is probable that these electrostatic interactions enhance ZCyt-ODN binding and account

for AdoMet's ability to increase the initial rate of formation of M.*Hha*I/ZCyt-ODN/AdoMet complexes relative to binary complexes or ternary complexes with AdoHcy. Since most of the ZCyt residues in ZCyt-ODN remain unmethylated in M.*Hha*I/ZCyt-ODN/AdoMet complexes, the measurable dissociation of these complexes (Table 2) suggests that at least some of the stabilizing effect of AdoMet on the initial complex of M.*Hha*I with ZCyt-ODN is lost once the flipped ZCyt is converted to form IIb. This is consistent with the observation that AdoMet has only a small stabilizing effect on complexes formed between M.*Hha*I and DZCyt-ODN.[37] Since the only difference between the structure of DZCyt and ZCyt-IIb in ODNs complexed with M.*Hha*I is the replacement of the proton at C6 of DZCyt with the covalent bond between ZCyt-IIb and Cys81, it seems likely that the slow but measurable dissociation of ZCyt-ODN from the ternary complex observed in our studies is related to the rate at which ZCytIIb is converted to either non-covalently bound form I or IVa. The observation that binary ZCyt-ODN complexes, which have the same closed conformation during native gel electrophoresis as ternary complexes with AdoMet, but dissociate ~three times faster (Table 2), suggests that even though the methyl group of AdoMet impairs its ability to stabilize intermediate IIb relative to AdoHcy, it can still affect the equilibrium between form IIb and forms I or IIIb or the conversion of IIIb to IVa.

SDS-PAGE as a tool for examining the determinants of stability of ZCyt-ODN/M.*Hha*I complexes

The primary criterion for identifying covalent bond formation between a C5-MTase and a cytosine analog in DNA is the ability of the DNA–enzyme complex to withstand heating at 95 °C in the presence of SDS and a reducing agent. Our studies and those of others[38] clearly demonstrate that C5-MTase complexes with ZCyt DNA do not withstand this treatment. Although our studies of the temperature stability of M.*Hha*I/ZCyt-ODN complexes in SDS and β-mercaptoethanol were undertaken to strengthen the evidence indicating that ZCyt in these complexes is covalently linked to M.*Hha*I, the results obtained in comparing M.*Hha*I-FCyt and ZCyt-ODN complexes also provided new structural information.

The first, and most surprising finding, was that both binary and ternary complexes formed by M.*Hha*I and ZCyt-ODN are not only stable to treatment with SDS and β-mercaptoethanol but that they are also more compact than unbound enzyme, i.e. they have a higher electrophoretic mobility than free M.*Hha*I during SDS-PAGE. The majority of M.*Hha*I/ZCyt-ODN complexes formed during incubation at 37 °C incubation are stable even after heating at temperatures >50 °C in the presence of 1% SDS and β-mercaptoethanol. Regardless of whether co-factor is present or not, the amount of radiolabeled ZCyt-ODN that remains associated with the rapidly migrating conformer of M.*Hha*I after treatment with SDS and β-mercaptoethanol at 25 °C is approximately equivalent to that detected in the closed complex after native gel electrophoresis. This is a strong argument for the conclusion that the rapidly migrating enzyme–ZCyt-ODN complex is the same as the closed complex detected in native gels. In contrast, FCyt-ODN complexes are completely stable to heating at 95 °C under denaturing conditions, but only a small fraction of the complexes migrate faster than free enzyme in SDS-PAGE gels at 22 °C. Essentially all ZCyt-ODN complexes do so (Figure 4). As expected, the majority of covalent M.*Hha*I/FCyt-ODN complexes migrate more slowly than unbound M.*Hha*I, although only a fraction of the population migrates as slowly as the completely denatured until heated to 95 °C. This differs markedly from M.*Hha*I/ZCyt-ODN complexes, which migrate at the same rate on SDS-PAGE regardless of the presence of co-factor or the temperature to which they were heated prior to loading for electrophoresis.

This behavior can be explained in terms of the major difference in the mechanism of formation of covalent complexes with ZCyt or FCyt in DNA. Formation of stable complexes between M.*Hha*I and FCyt-ODNs requires transfer of a methyl group to the FCyt target while methylation of the ZCyt target in ZCyt-ODNs is not only unnecessary but is actually a rare event relative to transfer of a proton. Thus, our results suggest that, at 22 °C, under denaturing conditions, the covalent complexes between enzyme and methylated FCyt-ODN take on a variety of conformations ranging from tightly closed to fully open because the presence of the methyl group destabilizes the complex. With increased temperature, the equilibrium is shifted to the fully open form (Figure 6 and A.S.B & J.K.C., unpublished data). It should be noted that at this stage of our investigations, we cannot determine which of the differently migrating forms of M.*Hha*I/FCyt-ODN/AdoHcy complex is analogous to the open complex in native gels. However, the finding that methylation destabilizes the interaction between a flipped cytosine and M.*Hha*I does not conflict with the X-ray crystallographic data indicating that M.*Hha*I assumes a fully closed complex with methylated covalently bound FCyt-ODN. It simply suggests that the conditions of close packing in crystals of M.*Hha*I ternary complexes favor stabilization of the closed complex. In modeling studies, O'Gara *et al.*[35] have observed that a tension develops between the methyl group on C5 of flipped MCyt and adjacent amino acid residues Pro80 and Cys81, which could destabilize the enzyme–DNA complex sufficiently to lead to release of the methylated product. Our results provide visual evidence that the closed conformation of covalently linked, methylated FCyt-ODN complexes is less stable to SDS-PAGE than the covalently linked

closed conformation of ZCyt-ODN complexes. This, in turn, suggest that SDS-PAGE gel electrophoresis provides a new tool for determining the stability of closed complexes between DNA methyltransferases and their flipped targets.

In this regard, it is of interest that M.*Hha* I/ZCyt-ODN complexes do not display multiple conformations during SDS-PAGE. The only form detected is the fully closed complex that migrates more rapidly than free enzyme. With heating, the amount of this complex is decreased and the amount of free ZCyt-ODN is increased but no open or denatured complexes are detected. This supports the proposal that methylation of ZCyt residues in DNA is a relatively rare event since there is no *a priori* reason based on structure to predict that methylated ZCyt would differ greatly from MCyt in its ability to decrease the stability of a closed complex. However, it leaves open the question of the nature of the process that leads to release of ZCyt-ODN from complexes heated under denaturing conditions. The data presented in Figure 8 indicate that the rate-limiting step for dissociation of M.*Hha* I/ZCyt-ODN is a temperature dependent process that is not influenced by co-factor. Evidence will be presented elsewhere (unpublished results) demonstrating that for a variety of targets that cannot form covalent bonds with M.*Hha* I both the structure of the target base and the presence or absence of co-factor strongly influence the ability of closed M.*Hha* I/ODN complexes to withstand heating under the conditions used for the SDS-PAGE analysis reported here. Thus, the simplest explanation for the results presented in Figure 8 is that the starting structure is the same in binary and ternary M.*Hha* I-ZCyt-ODN complexes (ZCyt-IIb) and that the rate-limiting step for thermal dissociation of complexes is loss of the covalent link to enzyme (ZCyt-IIb) either by heat-induced shift of equilibrium to ZCyt-I or ZCyt-IVa, rather than thermal denaturation of the enzyme. As discussed above, either pathway for conversion of ZCyt-IIb to a non-covalently bound form involves a change in the structure of the base that would lead to destabilization of the complex. While our results do not allow us to predict the effect of SDS, β-mercaptoethanol and heat on the balance between these two pathways, the end products of the two pathways are quite different. The one leads to release of intact ZCyt-ODN and unmodified enzyme, whereas the other leads to release of ZCyt-IVa ODN and formylated (inactive) enzyme. The effect of temperature on the balance between these pathways has significant consequences with regard to whether a potential mutagenic lesion is left in the genome of ZCyd or ZdCyd-treated cells.[30] Thus, it will be critical to determine whether and how much formylated enzyme is formed during the release of ZCyt-ODNS from M.*Hha* I and mammalian DNA MTases and how the kinetics of formation of formylated enzyme are affected by changes in temperature.

In summary, using a totally defined ZCyt-ODN, we have been able to demonstrate that in complexes formed in the presence of AdoMet, methylation is highly inefficient and that there is a slow but measurable release of a DNA cytosine (C5) methyltransferase, M.*Hha* I, from covalent linkage to ZCyt. Since AdoMet concentrations in mammalian cells are generally at least four-fold higher than those of AdoHcy,[51] this finding suggests that understanding the detailed mechanism of binding and release of mammalian cytosine C5 methyltransferases from ternary complexes with ODNs and AdoMet will be the next logical step in determining how to design better ODN inhibitors of DNA methyltransferases as anticancer drugs.

Materials and Methods

Design and synthesis of ODNs

All ODNs were prepared using conventional automated DNA synthesis. ODNs containing 5-methylcytosine, 5-fluorocytosine (FCyt) or furanose abasic (AP) residues ("dSpacer" phosphoramidite) were prepared by the UNMC/Eppley DNA Synthesis Core Facility using commercially available phosphoramidites (Glen Research). ZCyt phosphoramidites were synthesized, incorporated into ODNs and structures were confirmed by mass spectroscopy as described by Eritja *et al*.[45,52] The sequences of the ODNs used here are listed in Table 1.

Double-stranded 13-bp ODNs were formed by annealing the ss ODNs at 37 °C for 60 minutes. Double-stranded 24-bp ODNs with ZCyt residues were annealed by heating at 65 °C for ten minutes and slowly cooling to ~45 °C over a 60 minute period. All other 24 base-long ODNs were annealed by heating to 90 °C for ten minutes and slowly cooling to ~45 °C over a 60 minute period.

Assays of inhibitor methylation and inhibitory potency

Enzyme

M.*Hha* I was expressed from pHSHW-5 in *Eschericia coli* strain ER1727 and purified as described by Kumar *et al*.[53] The enzyme was >95% pure, migrating as a single band in Coomassie-stained polyacrylamide gels (Figure 4). The specific activity of the enzyme is 150,000 units/mg protein as defined by New England Biolabs. The concentration of enzyme in all assays is given in terms of moles protein as determined using the BioRad Coomassie G-250 assay with bovine serum albumin as standard. Under the assay conditions described below, 1 fmol of M.*Hha* I protein transfers 5 fmol methyl groups/minute to AMp:A′ (640 nM). The purified enzyme was a generous gift from Dr S. Kumar, New England Biolabs, Inc. and Dr X. Cheng, Emory University.

Analysis of methyltransferase inhibitors

To determine the effect of inhibitory ODNs on the rate of substrate methylation, sets of duplicate reactions (50 μl) in Methylation Reaction (MR) buffer (50 mM Tris (pH 7.5), 10 mM EDTA, 5 mM β-mercaptoethanol) containing 8.6 nM M.*Hha* I, 2.43 μM [^{3}H]AdoMet (specific activity 15 Ci/mmol, 1 Ci = 37 GBq) were initiated by

adding increasing concentrations of substrate (AM_p:A′) in the absence or presence of FCyt-ODNs (30 nM, 60 nM, or 120 nM) or ZCyt-ODNs (15 nM, 30 nM, and 60 nM). Reactions were incubated at 37 °C and terminated after five minutes, a time-point within the linear range of the assay. The amount of [^{3}H]CH_3 incorporated was determined as described.[11,37] Lineweaver–Burk plots of the resulting data were prepared to determine K_i values for FCyt and ZCyt-ODNs.

Methylation of ZCyt- or FCyt-ODNs

Sets of duplicate reaction mixtures (25 μl) in MR buffer contained 173 nM M.*Hha* I, 8 μM [^{3}H]CH_3-AdoMet (specific activity 84 Ci/mmol), and the indicated concentrations of ZCyt-ODN or FCyt-ODN over a range of 50–400 nM. These were incubated at 37 °C for 30 minutes, and [^{3}H]CH_3 incorporation measured as above. Plots of data were constructed using GraphPad Prism 3.0 software.

Analysis of binary and ternary M.*Hha*I-ODN complexes

Native gel shift assays

Binding reaction mixtures containing 45 nM ^{32}P-end-labeled ZCyt-ODN, 108 nM M.*Hha* I protein, 75 ng poly-(dAdT:dAdT), and 100 μM co-factor (as indicated in Figure) in M.*Hha* I binding buffer (50 mM Tris (pH 7.5), 10 mM EDTA, 5 mM β-mercaptoethanol, 13% (v/v) glycerol) were incubated at 22 °C for 30 minutes as described.[37] Unlabeled (4.5 μM) ZCyt-ODN (100-fold excess) was added to each binding reaction following the initial incubation and incubations continued at 22 °C for the indicated times. Complexes were analyzed by electrophoresis at 150 V for 2.5–3 hours on 10% (w/v) native polyacrylamide gels that had been pre-run at 100 V for one hour in TBE buffer (89 mM Tris borate (pH 8.0), 2 mM EDTA).

Dried gels were autoradiographed by exposure to Dupont Cronex 4 medical X-ray film for 1–24 hours. Band intensity was quantified by PhosphorImager (Molecular Dynamics) analysis. The percentage of ODN in complexes was determined for each lane by dividing the signal in the band containing complex by the sum of the signals from bands containing free ODN and ODN/enzyme complex and multiplying by 100. For dissociation analysis, the natural log of the percentage of ODN in complexes for each lane was calculated and plotted *versus* the time of competition. The (−) slope of these plots is equal to the k_{off}, and $t_{1/2}$ ($= 0.693/k_{off}$) was calculated.

SDS-PAGE binding assays

To assess the stability of complexes under denaturing conditions, radiolabeled ds-ODN, M.*Hha* I, and co-factor (concentrations indicated in captions) in M.*Hha* I binding buffer were incubated at 37 °C. After 60 minutes, reaction mixtures were brought to final concentrations of 10% (v/v) glycerol, 1% (w/v) SDS, and 1% (v/v) β-mercaptoethanol, heated at indicated temperatures for five minutes, loaded directly onto 10% SDS-polyacrylamide gels, and electrophoresed at 200 V for 60 minutes. Dried gels were analyzed as described above and then rehydrated in a solution containing 3% (v/v) glycerol, 30% (v/v) methanol and 10% (v/v) acetic acid to allow staining with Coomassie blue (GELCODE-Blue reagent, Pierce).

*Urea-PAGE analysis of ODNs released from M.*Hha *I complexes*

Binding reactions containing radiolabeled ZCyt-ODN, M.*Hha* I, and co-factor in M.*Hha* I binding buffer were incubated at 37 °C. After 60 minutes, samples were brought to a final concentration of 1% SDS and β-mercaptoethanol, 25% (w/v) xylene cyanole, 0.25% (w/v) bromophenol blue and 30% glycerol. After heating at 50 °C or 95 °C for five minutes, samples were loaded on a 15% acrylamide/8 M urea gel, and electrophoresed at 200 V for 30 minutes, the time required for the dye front to reach the bottom of the gel. Dried gels were subjected to autoradiographic analysis as described above.

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References

1. Chen, R. Z., Pettersson, U., Beard, C., Jackson-Grusby, L. & Jaenisch, R. (1998). DNA hypomethylation leads to elevated mutation rates. *Nature*, **395**, 89–93.
2. Baylin, S. B. (1997). Tying it all together: epigenetics, genetics, cell cycle, and cancer. *Science*, **277**, 1948–1949.
3. Li, E., Beard, C. & Jaenisch, R. (1993). Role for DNA methylation in genomic imprinting. *Nature*, **366**, 362–365.
4. Ehrlich, M., Gama Sosa, M. A., Huang, L. H., Midgett, R. M., Kuo, K. C., McCune, R. A. & Gehrke, C. (1982). Amount and distribution of 5-methylcytosine in human DNA from different types of tissues or cells. *Nucleic Acids Res.* **10**, 2709–2721.
5. Goelz, S. E., Vogelstein, B., Hamilton, S. R. & Feinberg, A. P. (1985). Hypomethylation of DNA from benign and malignant human colon neoplasms. *Science*, **228**, 187–190.
6. De Marzo, A. M., Marchi, V. L., Yang, E. S., Veeraswamy, R., Lin, X. & Nelson, W. G. (1999). Abnormal regulation of DNA methyltransferase expression during colorectal carcinogenesis. *Cancer Res.* **59**, 3855–3860.
7. Compans, R. W., Cooper, M., Ito, Y., Koprowski, K. H., Melchers, F., Oldstone, M., *et al.* (2000). *DNA Methylation and Cancer* Current Topics in Microbiology and Immunology (Jones, P. A., Vogt, P. K., *et al.*), Vol. **249**, Springer, La Jolla, CA.
8. el-Deiry, W. S., Nelkin, B. D., Celano, P., Yen, R. W., Falco, J. P., Hamilton, S. R. & Baylin, S. B. (1991). High expression of the DNA methyltransferase gene characterizes human neoplastic cells and progression stages of colon cancer. *Proc. Natl Acad. Sci. USA*, **88**, 3470–3474.

9. Liang, G., Salem, C. E., Yu, M. C., Nguyen, H. D., Gonzales, F. A., Nguyen, T. T., Nichols, P. W. & Jones, P. A. (1998). DNA methylation differences associated with tumor tissues identified by genome scanning analysis. *Genomics*, **53**, 260–268.
10. Malumbres, M., Perez de Castro, I., Santos, J., Fernandez Piqueras, J., Pellicer, A. & Malumbres, M. (1999). Hypermethylation of the cell cycle inhibitor p15INK4b 3′-untranslated region interferes with its transcriptional regulation in primary lymphomas. *Oncogene*, **18**, 385–396.
11. Creusot, F., Acs, G. & Christman, J. K. (1982). Inhibition of DNA methyltransferase and induction of Friend erythroleukemia cell differentiation by 5-azacytidine and 5-aza-2′-deoxycytidine. *J. Biol. Chem.* **257**, 2041–2048.
12. Christman, J. K., Mendelsohn, N., Herzog, D. & Schneiderman, N. (1983). Effect of 5-azacytidine on differentiation and DNA methylation in human promyelocytic leukemia cells (HL-60). *Cancer Res.* **43**, 763–769.
13. Zingg, J. M. & Jones, P. A. (1997). Genetic and epigenetic aspects of DNA methylation on genome expression, evolution, mutation and carcinogenesis. *Carcinogenesis*, **18**, 869–882.
14. Goldberg, J., Gryn, J., Raza, A., Bennett, J., Browman, G., Bryant, J. & Grunwald, H. (1993). Mitoxantrone and 5-azacytidine for refractory/relapsed ANLL or CML in blast crisis: a leukemia intergroup study. *Am. J. Hematol.* **43**, 286–290.
15. Pinto, A. & Zagonel, V. (1993). 5-Aza-2′-deoxycytidine (Decitabine) and 5-azacytidine in the treatment of acute myeloid leukemias and myelodysplastic syndromes: past, present and future trends. *Leukemia*, **7**, 51–60.
16. Rochette, J., Craig, J. E. & Thein, S. L. (1994). Fetal hemoglobin levels in adults. *Blood Rev.* **8**, 213–224.
17. Thibault, A., Figg, W. D., Bergan, R. C., Lush, R. M., Myers, C. E., Tompkins, A., Reed, E. & Samid, D. (1998). A phase II study of 5-aza-2′deoxycytidine (decitabine) in hormone independent metastatic (D2) prostate cancer. *Tumori*, **84**, 87–89.
18. Ravandi, F., Kantarjian, H., Cohen, A., Davis, M., O'Brien, S., Anderlini, P. *et al.* (2001). Decitabine with allogeneic peripheral blood stem cell transplantation in the therapy of leukemia relapse following a prior transplant: results of a phase I study. *Bone Marrow Transplant*, **27**, 1221–1225.
19. Momparler, R. L. & Ayoub, J. (2001). Potential of 5-aza-2′-deoxycytidine (Decitabine) a potent inhibitor of DNA methylation for therapy of advanced non-small cell lung cancer. *Lung Cancer*, **34**, S111–S115.
20. Wijermans, P., Lubbert, M., Verhoef, G., Bosly, A., Ravoet, C., Andre, M. & Ferrant, A. (2000). Low-dose 5-aza-2′-deoxycytidine, a DNA hypomethylating agent, for the treatment of high-risk myelodysplastic syndrome: a multicenter phase II study in elderly patients. *J. Clin. Oncol.* **18**, 956–962.
21. Schwartsmann, G., Schunemann, H., Gorini, C. N., Filho, A. F., Garbino, C., Sabini, G. *et al.* (2000). A phase I trial of cisplatin plus decitabine, a new DNA-hypomethylating agent, in patients with advanced solid tumors and a follow-up early phase II evaluation in patients with inoperable non-small cell lung cancer. *Invest. New Drugs*, **18**, 83–91.
22. Willemze, R., Suciu, S., Archimbaud, E., Muus, P., Stryckmans, P., Louwagie, E. A. *et al.* (1997). A randomized phase II study on the effects of 5-aza-2′-deoxycytidine combined with either amsacrine or idarubicin in patients with relapsed acute leukemia: an EORTC Leukemia Cooperative Group phase II study (06893). *Leukemia*, **11**, S24–S27.
23. Murad, A. M., Guimaraes, R. C., Amorim, W. C., Morici, A. C., Ferreira-Filho, A. F. & Schwartsmann, G. (1997). Phase II trial of paclitaxel and ifosfamide as a salvage treatment in metastatic breast cancer. *Breast Cancer Res. Treat.* **45**, 47–53.
24. Schwartsmann, G., Mans, D. R., Menke, C. H., Xavier, N., Caleffi, M., Ferreira Filho, A. F. *et al.* (1997). A phase II study of doxorubicin/paclitaxel plus G-CSF for metastatic breast cancer. *Oncology (Huntingt)*, **11**, 24–29.
25. Kantarjian, H. M., O'Brien, S. M., Keating, M., Beran, M., Estey, E., Giralt, S. *et al.* (1997). Results of decitabine therapy in the accelerated and blastic phases of chronic myelogenous leukemia. *Leukemia*, **11**, 1617–1620.
26. Christman, J. K. (1984). DNA methylation in friend erythroleukemia cells: the effects of chemically induced differentiation and of treatment with inhibitors of DNA methylation. *Curr. Top. Microbiol. Immunol.* **108**, 49–78.
27. Chatterji, D. C. & Gallelli, J. F. (1979). Stabilization of 5-azacytidine by nucleophilic addition of bisulfite ion. *J. Pharm. Sci.* **68**, 822–826.
28. Vesely, J., Cihak, A. & Sorm, F. (1968). Characteristics of mouse leukemic cells resistant to 5-azacytidine and 5-aza-2′-deoxycytidine. *Cancer Res.* **28**, 1995–2000.
29. Juttermann, R., Li, E. & Jaenisch, R. (1994). Toxicity of 5-aza-2′-deoxycytidine to mammalian cells is mediated primarily by covalent trapping of DNA methyltransferase rather than DNA demethylation. *Proc. Natl Acad. Sci. USA*, **91**, 11797–11801.
30. Jackson-Grusby, L., Laird, P. W., Magge, S. N., Moeller, B. J. & Jaenisch, R. (1997). Mutagenicity of 5-aza-2′-deoxycytidine is mediated by the mammalian DNA methyltransferase. *Proc. Natl Acad. Sci. USA*, **94**, 4681–4685.
31. Schluckebier, G., M, O. G., Saenger, W. & Cheng, X. (1995). Universal catalytic domain structure of AdoMet-dependent methyltransferases. *J. Mol. Biol.* **247**, 16–20.
32. Kumar, S., Cheng, X., Klimasauskas, S., Mi, S., Posfai, J., Roberts, R. J. & Wilson, G. G. (1994). The DNA (cytosine-5) methyltransferases. *Nucleic Acids Res.* **22**, 1–10.
33. Cheng, X., Kumar, S., Posfai, J., Pflugrath, J. W. & Roberts, R. J. (1993). Crystal structure of the *Hha*I DNA methyltransferase complexed with *S*-adenosyl-L-methionine. *Cell*, **74**, 299–307.
34. Klimasauskas, S., Kumar, S., Roberts, R. J. & Cheng, X. (1994). *Hha*I methyltransferase flips its target base out of the DNA helix. *Cell*, **76**, 357–369.
35. O'Gara, M., Klimasauskas, S., Roberts, R. J. & Cheng, X. (1996). Enzymatic C5-cytosine methylation of DNA: mechanistic implications of new crystal structures for *Hha*I methyltransferase–DNA–AdoHcy complexes. *J. Mol. Biol.* **261**, 634–645.
36. O'Gara, M., Horton, J. R., Roberts, R. J. & Cheng, X. (1998). Structures of *Hha*I methyltransferase complexed with substrates containing mismatches at the target base. *Nature Struct. Biol.* **5**, 872–877.
37. Sheikhnejad, G., Brank, A., Christman, J. K., Goddard, A., Alvarez, E., Ford, H., Jr *et al.* (1999). Mechanism of inhibition of DNA (cytosine C5)-methyltransferases by oligodeoxyribonucleotides

containing 5,6-dihydro-5-azacytosine. *J. Mol. Biol.* **285**, 2021–2034.
38. Gabbara, S. & Bhagwat, A. S. (1995). The mechanism of inhibition of DNA (cytosine-5-)-methyltransferases by 5-azacytosine is likely to involve methyl transfer to the inhibitor. *Biochem. J.* **307**, 87–92.
39. Friedman, S. (1986). Binding of the EcoRII methylase to azacytosine-containing DNA. *Nucleic Acids Res.* **14**, 4543–4556.
40. Santi, D. V., Norment, A. & Garrett, C. E. (1984). Covalent bond formation between a DNA–cytosine methyltransferase and DNA containing 5-azacytosine. *Proc. Natl Acad. Sci. USA*, **81**, 6993–6997.
41. Christman, J. K., Schneiderman, N. & Acs, G. (1985). Formation of highly stable complexes between 5-azacytosine-substituted DNA and specific non-histone nuclear proteins. Implications for 5-azacytidine-mediated effects on DNA methylation and gene expression. *J. Biol. Chem.* **260**, 4059–4068.
42. Friedman, S. (1985). The irreversible binding of azacytosine-containing DNA fragments to bacterial DNA(cytosine-5)methyltransferases. *J. Biol. Chem.* **260**, 5698–5705.
43. Beisler, J. A. (1978). Isolation, characterization, and properties of a labile hydrolysis product of the antitumor nucleoside, 5-azacytidine. *J. Med. Chem.* **21**, 204–208.
44. O'Gara, M., Horton, J. R., Roberts, R. J. & Cheng, X. (1998). Structures of *Hha* I methyltransferase complexed with substrates containing mismatches at the target base. *Nature Struct. Biol.* **5**, 872–877.
45. García, R. G., Brank, A. S., Christman, J. K., Marquez, V. E. & Eritja, R. (2001). Synthesis of oligonucleotide inhibitors of DNA (cytosine-C5) methyltranferase containing 5-azacytosine residues at specific sites. *Antisense Nucleic Acid Drug Dev.* **11**, 369–378.
46. Flatau, E., Gonzales, F. A., Michalowsky, L. A. & Jones, P. A. (1984). DNA methylation in 5-aza-2′-deoxycytidine-resistant variants of C3H 10T1/2 C18 cells. *Mol. Cell. Biol.* **4**, 2098–2102.
47. Klimasauskas, S., Szyperski, T., Serva, S. & Wuthrich, K. (1998). Dynamic modes of the flipped-out cytosine during *Hha* I methyltransferase–DNA interactions in solution. *EMBO J.* **17**, 317–324.
48. Klimasauskas, S. & Roberts, R. J. (1995). M.*Hha* I binds tightly to substrates containing mismatches at the target base. *Nucleic Acids Res.* **23**, 1388–1395.
49. Eritja, R., Adam, V., Avino, A., Diaz, A. R., Fabrega, C., Ferrer, E. *et al.* (1997). Preparation of Oligonucleotides containing non-natural base analogs. *Nucleosides Nucleotides*, **16**, 697–702.
50. O'Gara, M., Roberts, R. J. & Cheng, X. (1996). A structural basis for the preferential binding of hemimethylated DNA by *Hha* I DNA methyltransferase. *J. Mol. Biol.* **263**, 597–606.
51. Shivapurkar, N. & Poirier, L. A. (1983). Tissue levels of *S*-adenosylmethionine and *S*-adenosylhomocysteine in rats fed methyl-deficient, amino acid-defined diets for one to five weeks. *Carcinogenesis*, **4**, 1051–1057.
52. Eritja, R., Marquez, V. E. & Garcia, R. G. (1997). Synthesis and properties of oligonucleotides containing 5-aza-2′-deoxycytidine. *Nucleosides Nucleotides*, **16**, 1111–1114.
53. Kumar, S., Cheng, X., Pflugrath, J. W. & Roberts, R. J. (1992). Purification, crystallization, and preliminary X-ray diffraction analysis of an M.*Hha* I-AdoMet complex. *Biochemistry*, **31**, 8648–8653.
54. O'Gara, M., McCloy, K., Malone, T. & Cheng, X. (1995). Structure-based sequence alignment of three AdoMet-dependent DNA methyltransferases. *Gene*, **157**, 135–138.

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