Methylation inhibitors can increase the rate of cytosine deamination by (cytosine-5)-DNA methyltransferase

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ABSTRACT

The target cytosines of (cytosine-5)-DNA methyltransferases in prokaryotic and eukaryotic DNA show increased rates of C→T transition mutations compared to non-target cytosines. These mutations are induced either by the spontaneous deamination of 5-mC→T generating inefficiently repaired G:T rather than G:U mismatches, or by the enzyme-induced C→U deamination which occurs under conditions of reduced levels of S-adenosylmethionine (AdoMet) and S-adenosylhomocysteine (AdoHcy). We tested whether various inhibitors of (cytosine-5)-DNA methyltransferases analogous to AdoMet and AdoHcy would affect the rate of enzyme-induced deamination of the target cytosine by M.Hpa II and M.Sss I. Interestingly, we found two compounds, sinefungin and 5′-amino-5′-deoxyadenosine, that increased the rate of deamination 10 3-fold in the presence and 10 4-fold in the absence of AdoMet and AdoHcy. We have therefore identified the first mutagenic compounds specific for the target sites of (cytosine-5)-DNA methyltransferases. A number of analogs of AdoMet and AdoHcy have been considered as possible antiviral, anticancer, antifungal and anti-parasitic agents. Our findings show that chemotherapeutic agents with affinities to the cofactor binding pocket of (cytosine-5)-DNA methyltransferase should be tested for their potential mutagenic effects.

INTRODUCTION

S-Adenosylmethionine (AdoMet) is a ubiquitous methyl-donor for a wide variety of methyl-transfer reactions (1). Furthermore, AdoMet is the substrate required for the generation of decarboxylated AdoMet (deca-AdoMet) by AdoMet decarboxylase, which is the rate limiting step in the synthesis of spermine and spermidine (2). The importance of methyl-transfer and decarboxylation reactions has made them potential targets for chemotherapeutic intervention by competitive inhibition. A wide variety of analogs of AdoMet or its metabolites have been evaluated as potential inhibitors of the methyl-transfer or decarboxylation reactions (1–7). Other compounds interfere with the synthesis or degradation of AdoMet and influence the level of AdoMet, deca-AdoMet or its metabolites (8). Interference with the metabolism of AdoMet could have a variety of effects on the metabolism of a cell or could change the genomic DNA methylation pattern.

DNA methylation, which is known to function as a stable gene silencing mechanism (9–14), is essential for normal embryonic development since mouse embryos with the (cytosine-5)-DNA methyltransferase gene homozygously deleted are inviable (15). A methyl-donor deficient diet lowers the genomic DNA methylation level and increases the risk for liver and colon tumors in rats (16–18). The involvement of DNA methylation in the initiation or promotion of cancer has been well studied and altered DNA methylation patterns are found in many tumors (9–14). Changes in DNA methylation patterns could either induce the expression of oncogenes or silence the expression of tumor suppressor genes (9–14). Growth regulatory genes such as the p16 gene are inactivated by DNA methylation thus allowing continuous cell division required for tumorigenesis (13). Epigenetic changes of DNA methylation patterns are heritable and, since they occur with higher frequency than genetic changes, represent possible targets for altering the gene expression pattern in cancer cells (19).

Furthermore, since the target cytosine of the (cytosine-5)-DNA methyltransferase is mutated with a high frequency in bacteria and in human cancer and genetic disease, the methylation reaction confers not only the possibility of epigenetic changes but could also increase the rate of genetic changes (20–24). A high frequency of C→T transition mutations at CpG dinucleotides occurs in the p53 tumor suppressor gene in various tumor types and inherited diseases (Li-Fraumeni syndrome) (25). These mutations are assumed to arise either by the spontaneous or enzyme-mediated deamination of 5-mC→T in the absence of completely efficient repair systems, or by the (cytosine-5)-DNA methyltransferase induced deamination of C→U→T occurring at cofactor concentrations below the K m values of the enzyme for its cofactor AdoMet or its reaction product AdoHcy (26–31).

We have previously shown that 5-methylcytosine (5-mC) spontaneously deaminates at a rate 2–3-fold higher than C in double stranded DNA in a genetic reversion assay (32). Furthermore, in the absence of methyl donor AdoMet or reaction product AdoHcy, the rate of enzyme-mediated C→U→T transition
mutations is increased up to 10^4-fold when compared with the spontaneous rate in water (27). Mutations introduced into the AdoMet binding pocket of the M.HpaII (cytosine-5)-DNA methyltransferase that interfere with AdoMet binding and therefore mimic the absence of AdoMet also increase the deamination rate, even in the presence of cofactors (33).

We have now tested a group of compounds analogous to AdoMet or AdoHcy for their abilities to interfere with AdoMet in the methylation reaction of the bacterial (cytosine-5)-DNA methyltransferases M.HpaII and M.SssI. Furthermore, the effects of these compounds on DNA binding affinities of M.HpaII and M.SssI and their abilities to increase the rate of C→T transition mutations at the target sites of these methyltransferases in the presence and absence of AdoMet and AdoHcy were measured.

**MATERIALS AND METHODS**

**Bacteria**

The Escherichia coli strain ER2357 [endorA thi-1 supE44 mcr-67 ung-1 dut Darg-F-lac]U169 Δ(mcrC-mrr)114::IS10 recA1 F' proAB lacY1 ZAM15 zfd:Tn10(Tet') was kindly obtained from Dr Sha Mi (Cold Spring Harbor Laboratory).

**Materials**

S-Adenosylmethionine (AdoMet), M.HpaII and M.SssI DNA methyltransferases were purchased from New England Biolabs. S-Adenosylhomocysteine (AdoHcy), 5'-methylthio-5'-deoxyadenosine (MTA) and 5'-amino-5'-deoxyadenosine were purchased from Sigma. The syntheses of sinefungin, (S)-6-methyl-6-deaminosinefungin and 6-deaminosinefungin have been described previously (34). N8-Adenosyl-N4-methyl-2,4-diaminobutanoic acid was kindly provided by Dr A. Gall (Oridigm Corporation, Seattle) (35). All analogs were dissolved in water and concentrations confirmed spectrophotometrically (OD260). The TMP-5-fluoro-uracil phosphoramidite was purchased from Glen Research (Sterling, VA).

**Covalent interaction**

This assay is based on the covalent trapping of (cytosine-5)-DNA methyltransferase by the mechanism-based inhibitor 5-fluorocytosine present at the target C (36). The synthesis and labeling of the oligonucleotide used for covalent interaction has been described previously (33). The labeled oligonucleotide (20 ng) was incubated in 1× M.HpaII or 1× M.SssI buffer (New England Biolabs) and DNA methyltransferase (4 U M.HpaII; 2 U M.SssI) with 10 µM AdoMet and the various diluted cofactor analogs for 90 min at 37°C. Thereafter, SDS loading buffer was added and the samples were boiled for 5 min and loaded on a 10% SDS–PAGE according to Laemmli (37). The bands representing bound and free oligonucleotides were quantitated using a phosphorimager and the ratio of (bound)/(bound + free) was calculated. Experiments were performed twice with similar results.

**Gel mobility shift assay**

The labeling method of the oligonucleotides and the gel mobility shift assay have been described previously (38). A new non-specific competitor oligonucleotide was used that contained no CpG dinucleotide. Non-specific oligonucleotide:

Top strand: 5’-GGGCTCTATAGGACACCAACCATATGTT-3’

Bottom strand: 3’-CCCGAGTATCCGCGTGTTGCTGACTA-5’

Briefly, the DNA binding reaction was done by incubating DNA methyltransferase (4 U M.HpaII; 2 U M.SssI), 4 pmol of labeled oligonucleotides, 40 pmol of non-specific oligonucleotide in a 10 µl reaction buffer (50 mM Tris–HCl pH 7.5, 10 mM EDTA, 13% glycerol, 0.5 mM DTT and 0.2 µg/µl BSA) and 100 µM of the AdoMet analogs for 30 min at room temperature. After incubation the samples were electrophoresed on a 6% non-denaturing polyacrylamide gel for 2 h at 80 V. The bands representing bound and free oligonucleotides were quantitated using a phosphorimager and the ratio of (bound)/(bound + free) was calculated. The results shown represent the means of two experiments.

**Deamination assay**

The in vitro deamination assay was carried out as described previously (27,33). Briefly, 200 ng of the reporter plasmid CCGG-psV2-neo8 was incubated with the DNA methyltransferase (4 U M.HpaII; 2 U M.SssI) in 20 µl reaction buffer (for M.HpaII: 50 mM Tris–HCl pH 7.5, 10 mM EDTA, 1 mM DTT, 0.2 µg/µl BSA; for M.SssI: 50 mM Tris–HCl pH 7.5, 1 mM EDTA, 5 mM DTT, 50 mM NaCl, 0.2 µg/µl BSA, which is a buffer allowing processive methylation by M.SssI, since it contains no MgCl2; 39) for 16 h at 37°C. S-Adenosylmethionine or S-adenosylhomocysteine was added at 10 µM and the AdoMet analogs were serial diluted to the concentrations indicated in the figures. The plasmid DNA was extracted with phenol and chloroform and precipitated with ethanol using glycerol as a carrier. The DNA was transformed using an electroporator (Biorad) into electro-competent E.coli strain ER2357 which is deficient in restriction of 5-mC (mcrABC–) and in uracil glycosylase (ung–). A part of the bacteria was diluted and plated on ampicillin plates to score for the CCGG-pSv2-neo8 transformation efficiency and the remaining bacteria plated on kanamycin plates to score for CTGG-pSv2-neo8 revertants. The reversion frequency was calculated as (number of kanamycin resistant bacteria)/(number of ampicillin resistant bacteria). Experiments were performed at least twice with similar results.

**RESULTS**

**Experimental background**

Insight into the mechanism of (cytosine-5)-DNA methylation was recently obtained by the elucidation of the crystal structure of the bacterial methyltransferase M.Hhal in complex with an oligonucleotide containing the mechanism-based inhibitor 5-fluorocytosine at the target cytosine. This structure showed a flipped out cytosine covalently attached to the enzyme as a reaction intermediate (40). In the absence of AdoMet or AdoHcy, the transient formation of the covalent enzyme–cytosine intermediate is believed to be responsible for the enzyme-mediated C→U deamination occurring as a side reaction with a low rate constant of ∼5 × 10^-9/s at 37°C (27). Both AdoMet and AdoHcy are known to inhibit the deamination reaction (27), either by the rapid transfer of a methyl group, or by sealing the cofactor binding pocket from access to solvent water. Since a mechanism-
facilitate the deamination reaction (Fig. 1). Sinefungin (an evaluation of the chemical and structural requirements that out cytosine ring during the methylation reaction should allow for the moiety of AdoMet which is closely juxtapositioned to the flipped analogs of AdoMet and AdoHcy. Modification at the methionine studied the requirements for the deamination reaction by using stable amino-analog of AdoMet, diaminobutanoic acid (and structurally similar to AdoMet (34,35)). (analog 2, 3) inhibited the methylation reaction efficiently whereas the aminoanalog of AdoMet is necessary for covalent adduct formation and interference by AdoMet analogs prevents transfer thus giving a direct measure of inhibition of the methylation reaction. Therefore, in contrast to an assay only measuring competition with AdoMet for binding to the free enzyme, this assay measures the inhibition of the enzymatic reaction composed of competition with AdoMet, DNA binding and the methyl-transfer reaction. The assay was performed with the enzymes M.HpaII or M.SssI, that were incubated in the presence of AdoMet (10 µM) and various concentrations of cofactor analogs. In the case of M.HpaII the cofactor analogs with a complete methionine backbone (analogs 1, 2 and 3) inhibited the methylation reaction efficiently whereas the aminoanalog of AdoMet (4) and the cofactor analogs with a truncated methionine backbone (analogs 5 and 6) competed less efficiently with the methylation reaction (Fig. 2). In the case of M.SssI, inhibition of methylation by the analogs 1, 2 and 3 was strong, whereas the analogs 4 and 5 showed intermediate inhibition and the analog 6 weak inhibition (Fig. 2). For both enzymes AdoHcy competed slightly stronger than analog 1, in contrast to previously published findings with M.HhaI and M.EcoRI, in which the opposite result was found (45,46). In summary, the analogs tested can be roughly grouped into strong competitors (analogs 1, 2, 3) and analogs 4, 5 and 6) competed less efficiently with the methylation reaction (Fig. 2). In the case of M.SssI, inhibition of methylation by the analogs 1, 2 and 3 was strong, whereas the analogs 4 and 5 showed intermediate inhibition and the analog 6 weak inhibition (Fig. 2). For both enzymes AdoHcy competed slightly stronger than analog 1, in contrast to previously published findings with M.HhaI and M.EcoRI, in which the opposite result was found (45,46). In summary, the analogs tested can be roughly grouped into strong competitors (analogs 1, 2, and 3) containing the complete methionine backbone of AdoHcy, intermediate competitors (analogs 4, 5 and 6) and a weak competitor (analog 6 for M.SssI). The replacement of the carbon of analog 2 with the nitrogen in analog 4 rendered it a weaker competitor. Since analog 5 (MTA) inhibited DNA methylation by M.HpaII and M.SssI less strongly than AdoHcy, it is unlikely that it plays a major role in regulating DNA methylation in cancer cells, although such a role cannot be excluded completely (47,48).

Analogs of AdoMet compete with AdoMet for methylation

We first studied the abilities of the different cofactor analogs to bind to the AdoMet binding pocket and to interfere with AdoMet binding and the methylation reaction. A sensitive assay was used which is based on the covalent trapping of the (cytosine-5)-DNA methyltransferase to an oligonucleotide containing 5-fluorocytosine at the target cytosine (33,36). Methyl-transfer from AdoMet is necessary for covalent adduct formation and interference by AdoMet analogs prevents transfer thus giving a direct measure of inhibition of the methylation reaction. Therefore, in contrast to an assay only measuring competition with AdoMet for binding to the free enzyme, this assay measures the inhibition of the enzymatic reaction composed of competition with AdoMet, DNA binding and the methyl-transfer reaction. The assay was performed with the enzymes M.HpaII or M.SssI, that were incubated in the presence of AdoMet (10 µM) and various concentrations of cofactor analogs. In the case of M.HpaII the cofactor analogs with a complete methionine backbone (analogs 1, 2 and 3) inhibited the methylation reaction efficiently whereas the aminoanalog of AdoMet (4) and the cofactor analogs with a truncated methionine backbone (analogs 5 and 6) competed less efficiently with the methylation reaction (Fig. 2). In the case of M.SssI, inhibition of methylation by the analogs 1, 2 and 3 was strong, whereas the analogs 4 and 5 showed intermediate inhibition and the analog 6 weak inhibition (Fig. 2). For both enzymes AdoHcy competed slightly stronger than analog 1, in contrast to previously published findings with M.HhaI and M.EcoRI, in which the opposite result was found (45,46). In summary, the analogs tested can be roughly grouped into strong competitors (analogs 1, 2, 3) containing the complete methionine backbone of AdoHcy, intermediate competitors (analogs 4, 5 and 6) and a weak competitor (analog 6 for M.SssI). The replacement of the carbon of analog 2 with the nitrogen in analog 4 rendered it a weaker competitor. Since analog 5 (MTA) inhibited DNA methylation by M.HpaII and M.SssI less strongly than AdoHcy, it is unlikely that it plays a major role in regulating DNA methylation in cancer cells, although such a role cannot be excluded completely (47,48).

Analogs of AdoMet change the affinities of methyltransferases to their DNA targets

It has previously been shown that the binding of bacterial (cytosine-5)-DNA methyltransferases can be enhanced by either adding AdoHcy or by introducing a G:U mismatch at the target cytosine (38,49–51). It seemed possible that the cofactor analogs used here not only inhibited the enzymatic reaction by preventing the binding of AdoMet, but also increased or decreased the affinities of the enzymes to their target sequences. Therefore, the abilities of the various cofactor analogs to change the affinities of the enzymes M.HpaII and M.SssI to their target sequences was measured using gel mobility shift assays. The binding of M.HpaII was increased 3-fold by AdoHcy and ~2-fold by analog 2, but no stimulatory or inhibitory effects with analogs 1, 3, 4, 5 and 6 were observed (Fig. 3A). Introduction of a uracil instead of cytosine at

![Figure 1. Structures of analogs of AdoMet and AdoHcy used in this study. The chemical structure is drawn as under physiological conditions (pH 7.5) and the stereochemistry is indicated if known. The numbering 1–6 refers to the numbering used in the text.](image-url)
Figure 2. Competition of cofactor analogs with AdoMet in the methyl-transfer reaction. (A) Gel mobility shift assay using irreversible covalent binding of M.HpaII and M.SssI to an oligonucleotide containing 5-flurocytosine at the target cytosine. The concentration of AdoMet was constant at 10 µM, the concentration of cofactor analogs was between 8 and 500 µM. The numbering 1–6 refers to the cofactor analogs as described in Figure 1. The upper and lower bands show the enzymes in complex with double and single stranded oligonucleotides, respectively. (B) Graphic representation of the results from (A) as percentage of control without added analog after quantitating the counts with a phosphorimager.

the target site, thus generating a G:U mismatch, overrode the stimulatory effect of the cofactor (Fig. 3B).

The binding of the enzyme M.SssI was not only stimulated 3-fold by AdoHcy and analog 2, but also by analog 3 and weakly by analogs 1 and 5, suggesting that the two enzymes had different conformational requirements for the cofactor-mediated increase of binding to the target site (Fig. 3C). However, the introduction of a uracil instead of cytosine overrode the stimulatory effects of the cofactor, similar to the results with M.HpaII (Fig. 3D). It has been postulated that the presence of uracil at the target site increases the binding of the enzyme by lowering the energy required to flip out the target base into the catalytic pocket thus increasing the affinity to the target sequence (38,51). It is possible that some of the cofactor analogs induced a conformational change in the enzyme–DNA complex thus facilitating removal of the target base into the catalytic pocket. In conclusion, the inhibitory effects of the cofactor analogs on DNA methylation seen in Figure 2 were not a result of interference with binding of the methyltransferase–cofactor complex to DNA, but rather of interference with AdoMet binding and the methyl-transfer reaction.

Deamination in the presence and absence of AdoMet or AdoHcy

The results above show that the cofactor analogs all compete with different efficiencies with the methylation reaction and, depending on the analog and the enzyme, have stimulatory effects on DNA binding. We next investigated whether any of these structural and binding characteristics specific for each cofactor analog would influence the deamination rate, which so far has only been described to occur in the presence of decreased concentrations of AdoMet or AdoHcy (27–30). The plasmid CCGG-pSV2-neo6 was incubated with M.HpaII or M.SssI in the presence of 500 µM of each cofactor analog and 10 µM AdoHcy, a concentration that completely prevents the enzyme-induced deamination (27). The reversion frequency was measured by scoring the number of kanamycin resistant colonies harboring the reverted plasmid CTGG-pSV2-neo6. The presence of cofactor analog 1 led to a 102–103-fold increase of deamination to a reversion frequency similar to that in the absence of added AdoHcy (Fig. 4). Cofactor analog 6, which has been found to be a weak competitor with AdoMet (Fig. 2) also increased the reversion frequency, however, less efficiently than analog 1. Analogs 2, 3, 4 and 5 as well as AdoHcy did not lead to any increase of cytosine deamination. These analogs at 100 µM suppressed deamination when no AdoHcy was added (data not shown), except analog 5 (MTA), which only partially inhibited deamination probably since its affinity to the AdoMet binding pocket is low (Fig. 2).

We next measured the concentration dependency of the deamination reaction with the two analogs 1 and 6, in the presence of AdoMet or AdoHcy. A concentration dependent increase of deamination was observed with both enzymes M.HpaII and
Figure 3. Gel mobility shift assays in the presence of cofactor analogs (100 μM each). (A) Gel mobility shift assay with M.HpaII and the unmethylated target without mismatch and (B) with G:U mismatch. (C) Gel mobility shift assay with M.SssI and the unmethylated target without mismatch and (D) with G:U mismatch. All experiments are summarized on the right side, the standard deviation derived from two experiments is indicated by vertical lines. The numbering 1–6 refers to the cofactor analogs as described in Figure 1.

M.SssI (Fig. 5A). The reaction became saturated at higher concentrations, presumably because the concentrations of unmethylated plasmid or active enzyme became limiting during the assay. When compared with AdoHcy, the presence of AdoMet in the deamination reaction interfered weakly with cofactor analog 1 and strongly with analog 6. It can be assumed that the presence of AdoMet in our assay system leads to a gradual methylation of the reporter plasmid which is more pronounced with analog 6 which only weakly competes with AdoMet (Fig. 2). Although it has been shown that enzyme-mediated deamination can occur at the methylated target C, it occurs at a much lower rate than described here with the unmethylated target (29).

It was also interesting to measure whether the rate of enzyme-mediated deamination in the absence of AdoMet and AdoHcy would be increased by analogs 1 and 6. Analog 1 did not increase the reversion frequency by M.HpaII, whereas an up to 10-fold increase was observed with M.SssI (Fig. 5B). Analog 6 increased the reversion frequency with M.HpaII ~10-fold and up to 100-fold with M.SssI, leading to a maximum rate constant of ~5 × 10⁻⁹/s under our assay conditions.

DISCUSSION

Several steps involved in the enzymatic methylation reaction of cytosine in DNA have been recently clarified (52–56). Each (cytosine-5)-DNA methyltransferase has unique properties such as sequence specificity and the requirement of AdoMet or AdoHcy for efficient DNA binding, but all share a common
Figure 4. Deamination assay with M.\textit{Hpa}\textsubscript{II} and M.\textit{Sss}\textsubscript{I} in the presence of AdoHcy (10 µM) and of cofactor analogs 1–6 (each 500 µM). The experiment was performed in duplicate and the standard deviation is indicated by vertical lines. The numbering 1–6 refers to the cofactor analogs as described in Figure 1.

catalytic methyl-transfer mechanism (57). The target cytosine is flipped out from the double-helix after binding to the specific target sequence and positioned into the catalytic pocket of the enzyme (40). Nucleophilic attack at the C6 of cytosine by the thiolate of a conserved cysteine-residue of the enzyme activates the otherwise inert C5 of cytosine. The activated C5 attacks the electrophilic methyl group of AdoMet which is subsequently transferred to the cytosine moiety. Abstraction of a proton at C5 and dissociation of the covalent cysteine-C6 adduct completes the methyl-transfer reaction by β-elimination, thus forming 5-methylcytosine and AdoHcy. Since the methyltransferase–AdoHcy complex has weak affinity for the methylated target sequence, the complex rapidly dissociates.

A caveat to this reaction is the formation of the 5,6-dihydrocytidine intermediate in the absence of AdoMet or AdoHcy and the presence of solvent water that deaminates with increased frequency (58). In view of the high rate of transition mutations at CpGs in human cancer, it is unknown whether physiological or pathological conditions exist where the concentration of AdoMet as well as of AdoHcy is below the \( K_m \) value of the enzyme for its cofactors necessary for the deamination reaction. Considering the need to stably maintain the DNA sequence of an organism, the (cytosine-5)-DNA methyltransferases presumably have evolved in a way that the presence of substrate AdoMet and the reaction product AdoHcy both prevent the deamination reaction.

Our study was intended to gain further insight into the mechanism of cytosine deamination and into how such deaminations are prevented under normal physiological conditions. The affinity of the enzyme to the target sequence appears to play no significant role in the cofactor-mediated deamination since it occurs even in the absence of any cofactor (27) and since both cofactor analogs 1 and 6 that increase the rate of deamination do not change the binding affinities. Among the analogs suppressing the deamination (2, 3, 4, 5 and AdoHcy), only 2, 3 and AdoHcy increased the DNA binding, suggesting that the affinity of the

Figure 5. Concentration dependent increase in the deamination rate induced by M.\textit{Hpa}\textsubscript{II} and M.\textit{Sss}\textsubscript{I} with cofactor analogs 1 and 6. (A) In the presence of AdoHcy (10 µM) or AdoMet (10 µM) and cofactor analogs 1 and 6 (8–500 µM). (B) In the presence of cofactor analogs 1 and 6 (8–500 µM) without added AdoMet or AdoHcy. The numbering 1–6 refers to the cofactor analogs as described in Figure 1.
Figure 6. Comparison of the proposed mechanisms of (cytosine-5)-DNA methylation and cytosine deamination. The proposed intermediate of the reaction at the C5 of cytosine is shown on the left, the proposed intermediate of the reaction at the C4 of cytosine in the middle and the reaction product is shown on the right. 

(A) Bisulfite-mediated hydrolytic cytosine deamination (60–63); (B) enzyme-mediated cytosine deamination in the absence of any cofactor (27,52,64); (C) enzyme-mediated (cytosine-5)-DNA methylation in the presence of AdoMet (52–56); (D) suppression of enzyme-mediated cytosine deamination by AdoHcy (27,52), or cofactor analogs 2, 3, 4 and 5 (4 is shown); (E) enzyme-mediated cytosine deamination in the presence of cofactor analogs 1 and 6. E– stands for the enzyme donating a hydrogen, E-S– stands for the conserved cysteine-thiolate group of the enzyme.

enzyme to the target sequence does not play a role in preventing the deamination. Therefore, similar to the methylation reaction (59), the deamination reaction can be separated from the conformational changes required for target DNA binding.

Clues to the mechanism of cofactor analog mediated enzymatic deamination can be obtained by considering data obtained from both the chemical cytosine deamination mechanism and the enzymatic (cytosine-5)-DNA methylation mechanism (see Fig. 6 for a summary). The molecular mechanism of chemical deamination induced by chemicals such as bisulfite requires the formation of the unstable 5,6-dihydrocytidine intermediate, the protonation of N3 of cytosine and the presence of solvent water (Fig. 6 A; 60–63). Similarly it has been shown by measuring tritium exchange occurring at C5 of cytosine that the generation of the unstable 5,6-dihydrocytidine intermediate requires the nucleophilic attack of the enzyme-SH at C6 of cytosine and the presence of solvent water (Fig. 6 B; 52, 64). In the presence of AdoMet, the activated C5 of cytosine can accept the electrophilic methyl group from the cofactor but not a proton from the solvent (Fig. 6 C). In the presence of AdoHcy and possibly also of the analogs 2, 3, 4 and 5, there may be transient formation of the covalent intermediate, but since the presence of these cofactor analogs precludes accessibility of C5 of cytosine to protons of the solvent, it can only undergo reversal without proton exchange (Fig. 6 D; 52, 64). Both 1 and AdoHcy are known to stimulate the covalent binding of the methyltransferase to their target sites containing 5-azacytosine (65). However, the increase of deamination only occurred with 1 suggesting that covalent addition cannot be the only requirement for the deamination. Deamination also requires protonation at N3 mediated by the enzyme and at the activated C5 of cytosine, followed by hydrolytic attack at the activated C4 of the 5,6-dihydrocytidine intermediate by water (52). Analog 1 and 6 both contain an amino-group which is protonated and charged at physiological pH and is located at about the same position as the methyl group of AdoMet. Therefore, it is likely that proton exchange does not occur with the solvent, but rather with the charged amino group -(NH3)⁺ which is brought in by analogs 1 and 6 (Fig. 6 E). All the other analogs which do not induce deamination (analogs 2, 3, 4 and 5) are also devoid of chemical groups that can act as proton donors.

This mechanism of cofactor-mediated deamination therefore assumes that the charged amino group -(NH3)⁺ of analogs 1 and 6 serves as a hydrogen donor facilitating the formation of the 5,6-dihydrocytidine intermediate required for cytosine deamination. This mechanism is further supported by the observed stimulation of enzyme-mediated deamination by the analogs 1 and 6 in the absence of AdoMet or AdoHcy. For both enzymes the increase in deamination was higher with analog 6 than with analog 1, which is possibly due to the formation of a weaker DNA binding complex with analog 6 thus increasing the number of enzymatic attacks or to the more rapid replacement of the deprotonated with the protonated analog 6. In addition, it has been shown with the M.TaqI (adenosine-N6)-DNA methyltransferase (66) that the positively charged amino group of sinefungin takes the same position as the positively charged sulfur group of AdoMet, leading to a slightly different orientation in the pocket that might
allow the access of water, the hydrolytic attack at the C4 position of the 5,6-dihydropyrimidine intermediate and the completion of the deamination reaction. Interestingly, even the amino-analog of AdoMet (analog 4), which is charged and protonated at pH 7.5, neither leads to methylation nor to deamination, although it leads to hydrogen exchange with (uracil-5′)-DNA methyltransferase (67) and to methylation with homocysteine-5′-methyltetrahydrofolate methyltransferase (35). The bonding length and angle at N of analog 4 are different than at S of AdoMet leading to a slightly different conformation (35). Possibly, the requirements of each methyltransferase to the structure of the cofactor are different, with the (cytosine-5′)-DNA methyltransferases not allowing any structural deviations that could be mutagenic.

The abilities of the analogs 1 and 6 to increase the rate of cytosine deamination in the presence of AdoMet and AdoHcy with M.HpaII and M.SssI suggests that the same might occur with mammalian (cytosine-5′)-DNA methyltransferases that have a catalytic domain similar to the prokaryotic enzymes (68). The target C of the human (cytosine-5′)-DNA methyltransferase is mutated with 42-fold higher frequency both in the germ line and in tumors (10). It is not yet clear whether this high frequency of mutation is mainly due to the faster spontaneous deamination rate at 5-mC→T as compared with C or to the enzyme-mediated deamination leading to a C→U→T pathway of mutagenesis. Since G:T mismatches are less efficiently repaired than G:U mismatches, differences in repair efficiencies could also contribute to the increased mutation rate at the target site of (cytosine-5′)-DNA methyltransferase (69). It has been estimated that ~12 transition mutations at non-CpGs occur per generation in the human germ line (70). It can be assumed that some of these mutations escaped the repair by the uracil glycosylase initiated repair system, suggesting that an increase of the formation of uracil by (cytosine-5′)-DNA methyltransferase in the presence of cofactor analogs would also increase the accumulation of C→U→T transition mutations. There are several possibilities as to how uracil could escape repair by uracil glycosylase. We have previously shown that the bacterial DNA methyltransferase M.HhaI binds very strongly to target sites containing a G:U mismatch and blocks repair by uracil glycosylase (38, 51). The bacterial uracil DNA glycosylase recognizes uracil occurring in looped and damaged DNA inefficiently (71–73). Furthermore, if uracil is excised the result is an apyrimidinic site, which therefore could be directly involved in structuring the genome similar compounds in germ and somatic cells or in the environment.

Attempts to determine whether potential mutagenic agents such as nitric oxide or echinomycin might increase the rate of transition mutations at the CpG or 5-mCpG dinucleotides in double-stranded DNA have thus far not been successful (75, 76). Our findings add an additional endogenous or exogenous mechanism possibly responsible for the increased rate of transition mutations at the target site of (cytosine-5′)-DNA methyltransferases. An analog of AdoMet may possibly accumulate under certain physiological or pathological conditions thus competing with AdoMet or AdoHcy. 5′-Methylthio-5′-deoxyadenosine (analog 5, MTA), which accumulates in many tumor types (42, 43), did not increase the mutation rate significantly with the bacterial DNA methyltransferases. It is not known whether exposure to sinefungin (I) or 5′-amino-5′-deoxyadenosine (6) would lead to an increase of cytosine deamination. Sinefungin (I) is a naturally occurring antibiotic synthesized in the soil bacteria Streptomycyes griseolus and Streptomycyes incarnatus originally isolated from the Ivory Coast region in Africa (77, 78). It inhibits various methyltransferases including DNA methyltransferases and AdoMet decarboxylase (79–81). It has antiviral (80, 81), antifungal (77, 78), antiparasitic (82, 83) and anticancer (5, 47) activities, but to our knowledge it is not in use since it is nephrotoxic (84). It is not known how many bacterial species are synthesizing sinefungin or related compounds and how widespread such bacteria occur. However, it is possible that ingestion or infection with bacteria capable of synthesizing sinefungin would result in an increased deamination rate at CpGs in nearby cells of organisms containing (cytosine-5′)-DNA methyltransferase. 5′-Amino-5′-deoxyadenosine (6) is an inhibitor of adenosine kinase and has inhibitory activity against vaccinia virus (4, 85). It reduces the toxicity of 2′-deoxyadenosine-analogs by inhibiting their phosphorylation by adenosine kinase (86). A number of further analogs of AdoMet, deca-AdoMet and MTA have been considered as chemotherapeutic agents for the treatment of cancer and infectious disease (1–8, 87–91). Our findings indicate that such agents and their metabolites with potential affinity for the AdoMet binding pocket should be tested for their (cytosine-5′)-DNA methyltransferase mediated mutagenic action which is possibly not detectable with assays such as the Ames test (82).

Our findings could have implications for other biological systems where a high rate of C→T transition mutations at the target site of (cytosine-5′)-DNA methyltransferases have been observed. Possibly, the introduction of similar hydrogen donating groups into the catalytic center of (cytosine-5′)-DNA methyltransferase either as described here by analogs of AdoMet, by peptides, by amino acid modification or mutation would lead to methyltransferases with cytosine deaminase activity. Interestingly, repeated induced point mutations (RIP), a process generating C→T transition mutations at repeated sequences in the fungus Neurospora crassa, seems to have the same dinucleotide preference as the (cytosine-5′)-DNA methyltransferase (92). In organisms containing 5-methylcytosine in their genome, the depletion of the target sites of (cytosine-5′)-DNA methyltransferases could be both the result of the increased spontaneous deamination rate of 5-mC→T and the increased enzyme-mediated deamination rate of C→U→T due to enzymatic errors or to the presence of sinefungin and similar compounds in germ and somatic cells or in the environment. In higher eukaryotes, the (cytosine-5′)-DNA methyltransferases therefore could be directly involved in structuring the genome into CpG rich and CpG depleted regions by actively inducing C→U→T transition mutations, in addition to the mutations by the spontaneous 5-mC→T pathway.

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