ISSN 0006-2979, Biochemistry (Moscow), 2009, Vol. 74, No. 11, pp. 1175-1186. © Pleiades Publishing, Ltd., 2009.
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REVIEW

Inhibition of C5-Cytosine-DNA-Methyltransferases

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Received December 12, 2008
Revision received February 25, 2009

Abstract—Changes in the methylation pattern of genomic DNA, particularly hypermethylation of tumor suppressor genes, occur at early stages of tumor development. Errors in DNA methylation contribute to both initiation and progression of various cancers. This stimulates significant interest in searching for inhibitors of C5-DNA-methyltransferases (MTases). Here we review the known nucleoside mechanism-based reversible and irreversible inhibitors of the MTases, as well as non-nucleoside ones, and discuss their inhibitory mechanisms and application for MTase investigations and cancer therapy.

DOI: 10.1134/S0006297909110017

Key words: DNA methyltransferase, methylation pattern, carcinogenesis, gene inactivation, inhibitors of DNA-methyltransferases, mechanism of inhibition, cancer therapy

DNA methylation plays an important role in many cellular processes. In prokaryotes, it protects against foreign DNAs. It can also be implicated in regulatory processes such as control of DNA replication [1-3]. In eukaryotes, DNA methylation is a significant form of epigenetic information [4]. The DNA methylation status in mammalian cells is particularly determinative of their normal development, gene expression control, genome imprinting, and maintenance of chromatin structure.

DNA methylation is catalyzed by specific enzymes, DNA methyltransferases (MTases) [3, 5]. C5-MTases catalyze the transfer of a methyl group from a cofactor, S-adenosyl-L-methionine (AdoMet), to the C5 carbon atom of cytosine residue. During this process, AdoMet turns into S-adenosyl-L-homocysteine (AdoHcy). Bacterial MTases usually recognize palindromic 4-8-base nucleotide sequences in a DNA substrate [1]. In contrast, the eukaryotic MTases recognize the CpG sequence. Several thousand prokaryotic MTases [6] (see also http://rebase.neb.com) and three active eukaryotic MTases—Dnmt1 (the major form in mammalian cells), Dnmt3a, and Dnmt3b—are known to date [7].

Primary structures of both prokaryotic C5-MTases and catalytic domains of eukaryotic ones have 10 conserved motifs [5, 8, 9]. The motifs I, II, and X are responsible for AdoMet binding, motif IV contains amino acid residues implicated in the catalytic mechanism, and motifs VI and VIII participate in stabilization of the flipped out cytosine residue [10, 11]. A variable domain (Target Recognition Domain, TRD) situated between conservative motifs VIII and IX is responsible for specific contacts that MTases form with the recognition site of DNA [1, 7, 10, 12]. The TRD also contains residues required for stabilization of an intermediate complex formed in the course of methylation.

In mammalian cells the methylated CpG-regions are localized in a certain manner along DNA to form the methylation pattern [4]. This pattern is created *de novo* during embryogenesis and copied in each replication cycle. In some disorders, such as those associated with tumorogenesis, the methylation pattern is altered. Total hypomethylation of repeated DNA fragments causes genome instability in tumor cells [13]. Along with this, hypermethylation in primary tumors and tumor cell lines occurs due to the methylation of CpG-islands in promotor regions of certain genes, such as tumor suppressor genes, resulting in their inactivation during carcinogenesis [14-18].

Abbreviations: AdoHcy, S-(5′-adenosyl)-L-homocysteine; AdoMet, S-(5′-adenosyl)-L-methionine; d^{4H}Cyd, 1-(2′-deoxy-β-D-ribofuranosyl)-2-pyrimidinone; DTT, dithiothreitol; DZdCyd, 5,6-dihydro-5-aza-2′-deoxycytidine; EGCG, (−)-epigallocatechin-3-gallate; FdCyd, 5-fluoro-2′-deoxycytidine; MTase, DNA-methyltransferase; NEM, N-ethylmaleimide; P, 2-pyrimidinone; 4′-SdCyd, 4′-thio-2′-deoxycytidine; sinefungin, S-adenosyl-L-ornithine; XRSA, X-ray structural analysis; ZCyd, 5-azacytidine; ZdCyd, 5-aza-2′-deoxycytidine.

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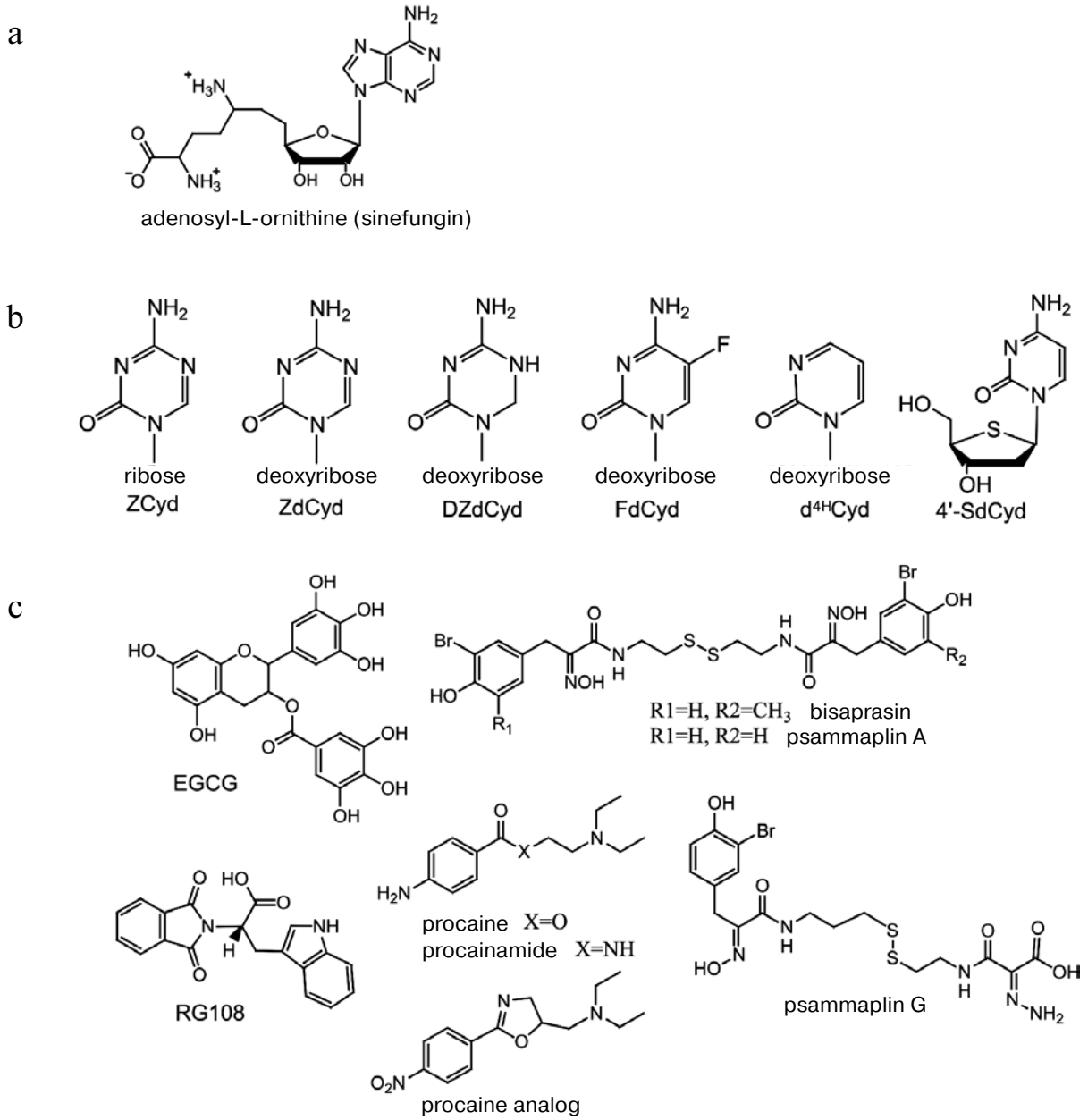
Unlike irreversible genome changes characteristic of tumor cells, hypermethylation of promotor regions as an epigenetic process can be reversible. It can be controlled by biochemical manipulations. This makes DNA methylation an attractive target for therapeutic intervention. Demethylation of tumor suppressor genes followed by their reactivation seems to be an advisable approach to therapy of malignant tumors. The DNA methylation status of the cell can be altered by influencing MTase activities using inhibitors of these enzymes. MTase inhibitors known to date can be divided into several groups. The first group comprises the reaction products: methylated DNA and AdoHcy. The second group comprises analogs of the cofactor AdoMet (AdoHcy) that are capable of binding with the enzyme but are not donors of a methyl group. The most widely used inhibitor of this type is sinefungin (S-adenosyl-L-ornithine) that is very similar to AdoMet in its structure (Scheme 1a) [2]. This group also comprises 5′-methylthio-5′-deoxyadenosine, 5′-amino-5′-deoxyadenosine, 5′-(2-carboxyethylthio)-5′-deoxyadenosine [19], etc. [20]. The third group comprises nucleoside inhibitors—2′-deoxycytidine analogs [21] (Scheme 1b).



DNA methyltransferase inhibitors: a) AdoMet analog; b) analogs of target dCyd; c) low-molecular-weight substances

Scheme 1

This group includes 5-azacytidine (ZCyd, vidaza) [22], 5-aza-2′-deoxycytidine (ZdCyd, decitabine) [22-24], 5,6-dihydro-5-aza-2′-deoxycytidine (DZdCyd) [22, 25], 5-fluoro-2′-deoxycytidine (FdCyd) [26], 1-(2′-deoxy-β-D-ribofuranosyl)-2-pyrimidinone (d^{4H}Cyd, zebularine) [27-29], and 4′-thio-2′-deoxycytidine (4′-SdCyd) [30]. All of these are mechanism-based inhibitors. They only exhibit inhibitory activity being specifically incorporated into a DNA sequence. The fourth group comprises specific single-stranded oligonucleotides and DNA duplexes (analogs of natural MTase substrates possessing higher affinity to MTases in comparison with the natural substrates) [31-33]. The fifth group comprises various low molecular weight non-nucleoside substances (Scheme 1c) such as nutritional polyphenols isolated from green tea, particularly (−)-epigallocatechin-3-gallate (EGCG) [34], and soybean [35]; disulfide derivatives of L-bromotyrosine (psammaplins) [36]; the L-tryptophan derivative RG108 [37, 38]; the 4-aminobenzoic acid derivatives procaine (2-(diethylamino)ethyl-4-aminobenzoate), procainamide (4-amino-N-[2-(diethylamino)ethyl]benzamide) [39-41], and their analogs [42]; mitramycin A [43]; and DNA intercalators such as doxorubicin [44] and echinomycin [45].

One should bear in mind that the effect of MTase inhibitors might be non-selective. MTase inhibitors can cause (along with activation of tumor suppressor genes) undesirable activation of some other genes (proto-oncogenes, genes associated with invasion and metastasis, imprinted genes, and so on) and promote hypomethylation of repeated DNA sequences and elevation of genome instability [46, 47]. The problems arising with DNA demethylation are considered in review [48].

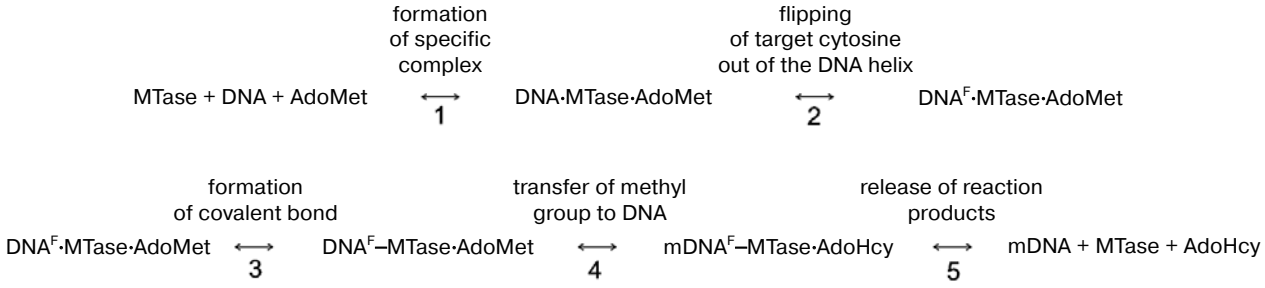
In the present review we focus on nucleoside MTase inhibitors as well as on the most discussed low molecular weight inhibitors. We look into methylation inhibition mechanisms, application of inhibitors for studying MTases, as well as examples of MTase inhibitor use in epigenetic cancer therapy.

NUCLEOSIDE ANALOGS OF TARGET 2′-DEOXYCYTIDINE (MECHANISM-BASED INHIBITORS)

Among MTases, the prokaryotic MTase M.HhaI (the recognition site is G<u>C</u>GC, the cytosine undergoing methylation is underlined) is the best studied to date. The methylation mechanism is complex and multistage [49]; the main stages are presented in Scheme 2. Initially, the enzyme binds to the DNA substrate and AdoMet to form a specific MTase·DNA·AdoMet complex (stage 1). Then the target cytosine residue flips out of the DNA double helix (stage 2). Subsequently, the covalent intermediate MTase–DNA·AdoMet is formed (stage 3). Then follows the methyl group transfer to DNA and dissociation of the complex into the reaction products (stages 4 and 5).

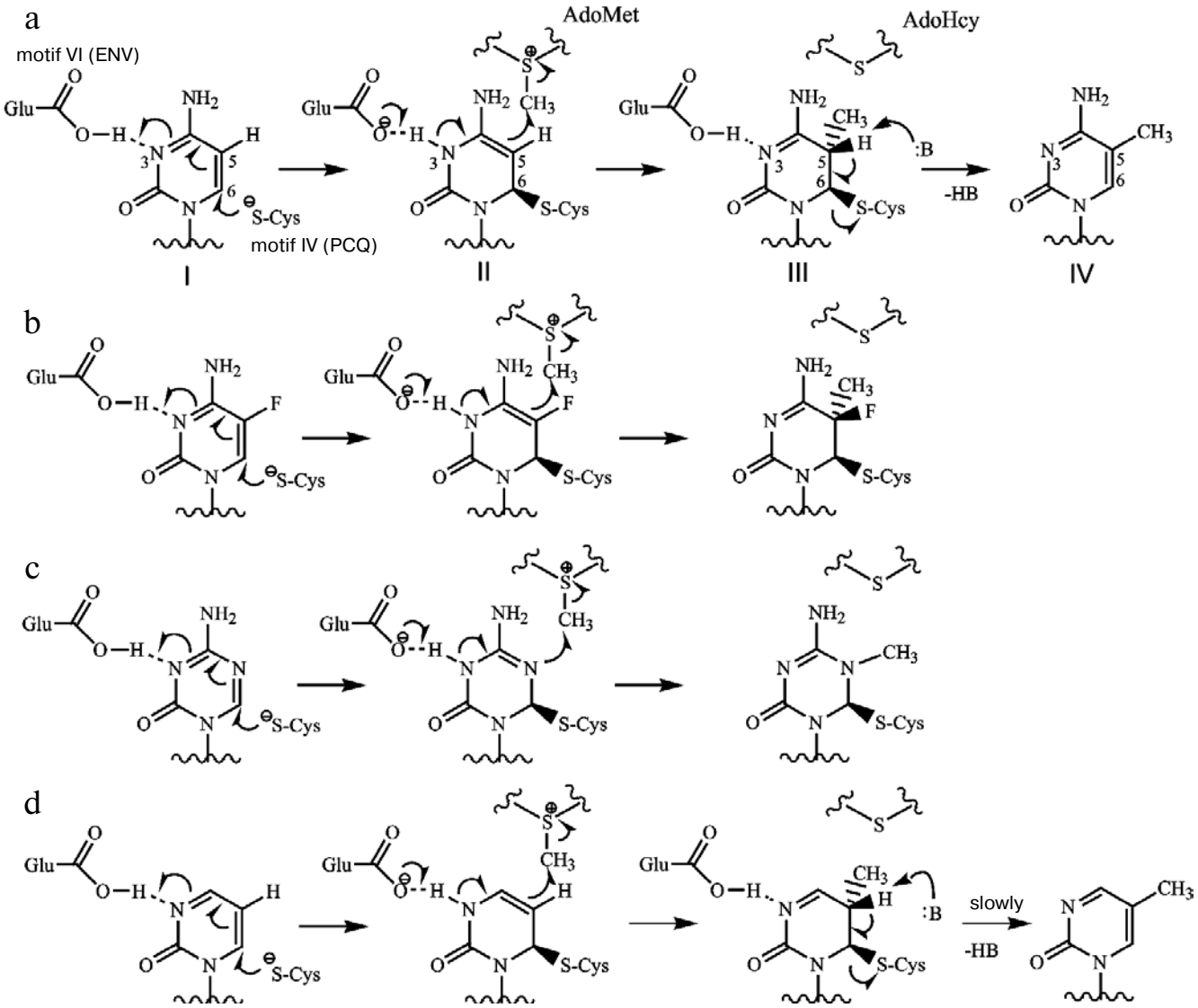
It has been demonstrated using M.HhaI as an example that MTases are not specific to the flipped out target cytosine and do not form specific contacts with it [3, 50]. This allows application of synthetic oligonucleotides containing nucleoside analogs at the position of the residue to be methylated (target 2′-deoxycytidine, dCyd) for structural and biochemical investigations. Moreover, this also finds relevance in clinical practice because gives a possibility for design of novel inhibitors, target dCyd analogs, which can be used for prevention of aberrant DNA-methylation.

The mechanism of methyl group transfer to DNA by MTases proposed by Wu and Santi [51] was later detailed [52] (Scheme 3a). The key reaction step is formation of a covalent MTase–DNA intermediate. Nucleophilic attack by the thiol group of a cysteine residue from the MTase conservative PCQ motif (motif IV) on the C6 carbon atom of the cytosine residue to be methylated leads to appearance of the covalent bond between the cysteine residue and the target cytosine. The covalent intermediate formation together with protonation of the N3 position of cytosine, in which glutamic acid from the conserved ENV motif (motif VI) is implicated, leads to activation of C5 atom for electrophilic attack. Then the methyl group is attached at C5 position of cytosine fol-



Mechanism of DNA methylation by M.HhaI. Designations: MTase, M.HhaI; DNA^F, DNA substrate with flipped-out cytosine; $mDNA^F$, methylated DNA substrate with flipped-out cytosine; mDNA, methylated DNA, the reaction product; • and –, non-covalent and covalent bonds, respectively

Scheme 2



DNA methylation mechanism of MTases when DNA substrates contain cytosine (natural DNA substrate) (a) and mechanism-based inhibitors FCyt, ZCyt, and d^{4H}Cyt (b-d), respectively [23, 26, 29, 58], at position of target cytosine

Scheme 3

lowed by β-elimination of the conserved cysteine residue of the MTase.

The following nucleoside analogs of target dCyd have been used to date: ZCyd, ZdCyd, FdCyd, and d^{4H}Cyd (Scheme 1b); being specifically incorporated into DNA, they affect the stage of covalent MTase–DNA intermediate formation. Such inhibitors were named mechanism-based. Despite its inability for covalent linkage to MTase, DZdCyd is also a mechanism-based inhibitor. Another known inhibitor, the target dCyd analog 4′-SdCyd, is also occasionally [28] named mechanism-based.

Irreversible inhibitors. Design of irreversible mechanism-based inhibitors was based on the search for target dCyd modifications counteracting or significantly decelerating dissociation of the MTase–DNA conjugate. The found modifications were substitutions of hydrogen atom at C5 position by fluorine (FdCyd), substitution of C5 carbon by nitrogen (ZCyd and its deoxy-analog ZdCyd), and removal of exocyclic amino group in the target dCyd (d^{4H}Cyd) (Scheme 1b).

FdCyd, ZCyd (ZdCyd), and d^{4H}Cyd were shown to inhibit DNA methylation in mammalian cells and influence gene expression and cell differentiation [53-56]. For instance, d^{4H}Cyd stimulated differentiation of clonal murine embryo cell line 10T1/2 to muscle cells [55] and even was capable of providing trans-differentiation from myoblasts to smooth muscle cells [56].

The mechanisms of MTase inhibition by FdCyd, ZCyd (ZdCyd), and d^{4H}Cyd are exactly alike. In 1984,

Santi and associates proposed a molecular mechanism for MTase inhibition by target dCyd analogs, particularly FdCyd and ZCyd [57], which can now be presented as displayed in Scheme 3 (b and c, respectively) [23, 26, 29]. Initially, nucleophilic attack by a Cys residue of the enzyme on the C6 position of the pyrimidine ring of target dCyd analog occurs, which is facilitated by protonation of the N3 position with involvement of a Glu residue. In the presence of AdoMet, the methyl group is transferred to position 5. The formed MTase–DNA intermediate remains stable because the stage of β-elimination does not occur, which causes irreversible inactivation of the enzyme. The molecular mechanism of MTase inhibition by d^{4H}Cyd (Scheme 3d) resembles that for both FdCyd and ZCyd, but in this case inactivation of MTases is conditionally irreversible because of gradual decay of the conjugate [58].

The inhibitory ability of FdCyd is explained by the presence of fluorine instead of hydrogen at position C5 of dCyd, whereas a proton has to be removed in the course of the reaction. The fluorine atom is the most conservative substitution of a hydrogen atom in organic substances [59]. However, unlike hydrogen that can be eliminated in the form of a proton, the fluorine does not form the corresponding cation (F^+). The nature of inhibition by ZCyd and d^{4H}Cyd is quite different. These inhibitors are characterized by increased reactivity of the C6 position. In both cases (for d^{4H}Cyd it has been demonstrated by calculation of molecular orbitals of 2-pyrimidinone (P) and cytosine [29]) the cause of activation of the cytosine ring C6 position is polarization of the C5–C6 double bond due to either presence of a nitrogen atom at position 5 in ZCyd or removal of an electron donor amino group (NH_2) from the C4 position in d^{4H}Cyd. These modifications lead to a deficit of electrons in the system and to increase in positive charge at position 6 [23, 27, 29]. This facilitates interaction of ZCyd and d^{4H}Cyd with the thiol group of a highly conserved Cys residue of the enzyme and can promote nucleophilic attack by weaker nucleophiles such as OH-groups of Ser or Thr [28] because of lowered reaction energy barrier. Unlike dCyd, the reactivity of the C6 position in FdCyd remains unchanged, so the reaction does not occur with Ser or Thr [28].

It should be noted that the affinity of MTases to d^{4H}Cyd-DNA is considerably higher than that to unmodified DNA because of the absence of one hydrogen bond in the P·G pair compared with the Watson–Crick pair C·G. This allows the flipping of P and formation of a tight enzyme–substrate complex, that is, it elevates the efficiency of MTase inhibition [27].

Formation of covalent intermediates of various MTases with FdCyd, ZCyd (ZdCyd), and d^{4H}Cyd-DNA was commonly observed by SDS-PAGE (table). The covalent intermediates with FdCyd-DNA were isolated and characterized for M.HaeIII, M.EcoRII, and human MTase [60-62], and later for M.HhaI [10].

Stability of MTase conjugates with FdCyd, ZCyd (ZdCyd), and d^{4H}Cyd-DNA is highly dependent on both the nature of the target dCyd analog and the presence of AdoMet (table). In all cases, conjugates formed in the presence of AdoMet were stable under treatment with SDS. However, the ternary complexes MTase–(FdCyd-DNA)·AdoMet were resistant to 1% SDS at 95°C [26], whereas the complexes MTase–(ZdCyd-DNA)·AdoMet and MTase–(d^{4H}Cyd-DNA)·AdoMet only held at temperatures to 70°C [23, 58], that is, stability decreased in the order FdCyd > ZdCyd ~ d^{4H}Cyd. In the absence of AdoMet, FdCyd-DNA plays a role of competitive inhibitor with regard to unmodified DNA to form a noncovalent enzyme–substrate complex with, for instance, M.HhaI (K_i ~ 3 nM) [26]. In the absence of AdoMet, ZCyd(ZdCyd)-DNA formed a conjugate with M.EcoRII, which was less stable than that formed in the presence of AdoMet [23]. As for d^{4H}Cyd, stability of conjugates formed in the absence of AdoMet differed for different MTases. For example, M.MspI formed conjugates with d^{4H}Cyd-DNA that were equally stable both in the absence and presence of AdoMet, whereas stability of conjugates between M.HgaI-2 and d^{4H}Cyd-DNA decreased under similar conditions [28].

An important point is that the transfer of a methyl group to DNA is possible for all modified DNAs under consideration (in accordance with Scheme 3, b-d), but then the reaction either stops (Scheme 3, b and c) [29] or extremely decelerates (Scheme 3d) [58]. It is likely that the methyl group transfer by MTases on FdCyd- and ZCyd-DNA only occurs during the first reaction cycle [23], whereas in the case of d^{4H}Cyd-DNA the transfer of methyl groups is also possible during following cycles. From a chemical point of view, the presence of a methyl group at the C5 position should not lessen the reactivity of the heterocycle C6 position in conjugate formation. For instance, M.MspI covalently attaches to DNA containing 5-methyl-2-H-pyrimidinone, although weaker than to d^{4H}Cyd-DNA [28].

Reversible inhibitors. Reversible mechanism-based inhibitors, like irreversible ones, were constructed by introduction of modifications into the target dCyd. A good example of this type inhibitors is 5,6-dihydro-5-aza-2′-deoxycytidine (DZdCyd) carrying an sp^3-hybridized carbon atom at position 6 and an NH-group at position 5 of the pyrimidine ring (Scheme 1b). Inhibitory properties of DZdCyd-DNA are due to formation of a stable enzyme–substrate complex with MTases simulating the methylation reaction intermediate in which the cytosine residue to be methylated turns into dihydrocytosine. In this case, DZdCyd-DNA does not form a covalent linkage with the enzyme [25].

Characteristic features of the MTase·(DZdCyd-DNA) complex were explored using M.HhaI as an example. Local disruption of DNA base stacking in the locus of DZdCyd incorporation did not influence the DNA

Conjugates of MTases (or their mutant forms) with DNAs containing mechanism-based inhibitors and properties of these conjugates

Enzyme	Inhibitor	Cofactor/analog	Properties of conjugates*	Reference
M.HhaI	poly(FdCyd-dG)	AdoMet	stable in 1% SDS at 95°C	[26]
M.HhaI, E119A, C81A	FdCyd at position of target dCyd in 30-mer hemi-methylated DNA duplex	AdoMet	stable in 12% SDS at 95°C; E119A and C81A do not form conjugates	[92]
M.EcoRII	ZCyd at position of target dCyd in 25-mer hemi-methylated DNA duplex	AdoMet, AdoHcy or in their absence	stable in 0.8% SDS at 70°C in presence of AdoMet, at 37°C in presence of AdoHcy, or in their absence	[23]
M.EcoRII	FdCyd-containing DNA	AdoMet	stable at 100°C	[93]
M.EcoRII, C186S/W	FdCyd at position of target dCyd in 14-mer hemi-methylated DNA duplex	AdoMet, AdoHcy	stable in 0.1% SDS in presence of AdoMet; stability considerably decreases in presence of AdoHcy; C186S/W do not form conjugates	[78]
M.EcoRII, C186S/A	ZCyd at position of target dCyd in 25-mer hemi-methylated DNA duplex	AdoMet	stable in 0.8% SDS at 70°C; C186S is stable in 0.8% SDS, but does not sustain heating; C186A does not form conjugates	[79]
M.EcoRII	d^{4H}Cyd at position of target dCyd in 14-mer DNA duplex	AdoMet, AdoHcy	stable in 1% SDS at 65°C; decays when heated to 90°C	[58]
M.Dcm, C177S	FdCyd at position of target dCyd in 15-mer DNA duplex	AdoMet, sinefungin	stable in 2% SDS at 100°C; C177S does not form conjugates	[94]
M.HaeIII	FdCyd at position of target dCyd in DNA duplex	AdoMet	stable in 1% SDS at 95°C	[60]
M.MspI C174S/T/Y	d^{4H}Cyd at position of target dCyd in 30-mer DNA duplex	sinefungin	stable in 1% SDS**; C174Y does not form conjugates	[28]
M.HgaI-2	d^{4H}Cyd at position of target dCyd in 19-mer DNA duplex	sinefungin	stable in 1% SDS**; do not sustain SDS-PAGE	[28]
Catalytic domain of Dnmt3a and its mutants C120A and E166A	FdCyd at position of target dCyd in 30-mer DNA duplex	AdoMet	stable in 2% SDS at 90°C; C120A does not form conjugates	[95]
Dnmt2	FdCyd at position of target dCyd – simultaneously in three CG-sites of 80-mer DNA duplex	AdoMet	stable in 2% SDS at 65°C	[96]

* SDS-PAGE analysis, if another is not specified.
** PAGE analysis under non-denaturing conditions.

recognition by M.HhaI, either in the absence or presence of AdoHcy: stable (DZdCyd-DNA)·M.HhaI complexes were formed under non-denaturing conditions. Electrophoretic mobility of these complexes corresponded to the "closed" M.HhaI conformation in which the active site loop encloses the flipped base within the catalytic cavity of the enzyme. The complexes decayed on heating to 95°C in the presence of either SDS or dithiothreitol (DTT). It has been demonstrated that DZdCyd occupying the position of the target dCyd is not methylated by MTases [25].

Another type of reversible mechanism-based inhibitors is 4′-thionucleosides such as 4′-thio-2′-deoxycytidine (4′-SdCyd, Scheme 1b). Replacement of the oxygen atom with a sulfur atom in the carbohydrate moiety had virtually no effect on the structure, but significantly influenced the biological functions of DNA [30].

It has been shown using M.HhaI as an example, that 4′-SdCyd incorporated into the recognition site instead of the target dCyd considerably inhibited the MTase catalytic activity. Kinetic analysis revealed the competitive character of M.HhaI inhibition by 4′-SdCyd-DNA with regard to unmodified DNA (K_i ~ 2-5 nM). It has been supposed that the inhibitory effect resulting from introduction of a sulfur atom into position 4′ develops after the

"flipping out" stage but before the methyl group transfer. The cause of inhibition is that, due to modification of deoxyribonucleic ring, the orientation of the target cytosine becomes improper for nucleophilic attack by the MTase Cys residue. So, the covalent intermediate formation seems to become impossible. Moreover, it has been supposed that the replacement of O4′ with S4′ atom possessing longer Van der Waals radius breaks functionally important DNA–protein contacts.

Application of mechanism-based inhibitors for epigenetic therapy of cancer. Tumor cells are characterized by hypermethylation of promoter regions of certain genes, such as tumor suppressor genes, leading to their inactivation during carcinogenesis [14-18]. Mechanism-based MTase inhibitors are promising pharmaceuticals for epigenetic therapy of malignant tumors because they allow for correction of excessive activity of MTases. Experiments *in vivo* on human tumor cell lines have shown that FdCyt, ZCyd (ZdCyd), and d^{4H}Cyd can cause demethylation and reactivation of genes blocked due to methylation.

ZCyd (Vidaza) and ZdCyd (decitabine) synthesized more than 40 years ago [22, 63] are widely used as effective chemotherapeutic agents against malignant blood diseases, in particular myelodysplastic syndrome (MDS) and other leucoses [64].

The mechanisms of antitumor activity of ZCyd and ZdCyd *in vivo* are not yet well understood [24]. In accordance with current views, they reactivate hypermethylated genes, particularly those implicated in cell cycle regulation (such as *p53*), due to inhibition of cellular MTases [24, 65]. Also, formation of conjugates between MTases and ZdCyd-containing genomic DNA leads to DNA damage and is accompanied by cytotoxic effects [24, 66]. On the whole, this results in cell cycle arrest or apoptosis [24, 65]. For instance, the treatment of tumor cell lines HeLa and HCT116 with ZdCyd decreases Dnmt1, Dnmt3a, and Dnmt3b MTase levels in soluble nuclear fraction, inhibits cell growth, leads to the cell cycle arrest at the G2 phase, reduces colony-forming capability, and causes (either directly or indirectly) DNA double-strand breaks.

ZCyd and ZdCyd are accepted as effective MTase inhibitors; however, they have some significant faults, primarily high cytotoxicity. The fact is that in living cells uridine-cytidine kinases convert ZCyd and ZdCyd into monophosphates, which then become triphosphates. In this form, ZCyd is incorporated into RNA and DNA, respectively, resulting in inhibition of biosynthesis of DNA, RNA, and proteins in cells [22]. ZdCyd (like FdCyd and d^{4H}Cyd) [67] can only be incorporated into DNA. Nonetheless, it is one order of magnitude more toxic than is ZCyd. Also, both ZCyd and ZdCyd are unstable in aqueous solutions because of hydrolysis, which makes impossible their application in oral form, and can undergo deamination by cellular deaminases to form 5-azauridine and 5-aza-2′-deoxyuridine, respectively. To stabilize ZdCyd, authors [68] attempted to use dinucleotides with incorporated ZdCyd in experiments *in vivo*. This recipe does not influence the virtue, but decreases deamination of ZdCyd.

In the ZCyd analog DZdCyd, the linkage between the atoms at positions 5 and 6 is saturated. This precludes nucleophilic attack by a water molecule on the C6 position and results in higher resistance to hydrolysis of DZdCyd as compared with ZCyd [69]. However, clinical trials of DZdCyd were not very successful because it cannot be phosphorylated by cytidine kinase *in vivo* [70].

Unlike ZCyd and ZdCyd, d^{4H}Cyd (zebularine) is characterized by low cytotoxicity and high stability in aqueous solutions [71]. A long treatment of urinary bladder carcinoma cells with d^{4H}Cyd resulted in growth suppression, induction, and maintenance of expression of the *p16* gene, which was hypermethylated, and maintenance of demethylated status of the 5′-region of this gene for 40 days [72]. d^{4H}Cyd also induced demethylation of other hypermethylated regions, particularly those characterized by low contents of CpG sequences. Suppression of cell growth was also observed in six other human tumor cell lines: HCT15, CFPAC-1, SW48, HT-29, PC3, and CALU-1 [73]. It is of great importance that similar treatment of normal human fibroblasts did not lead to significant suppression of their growth. It was found that d^{4H}Cyd considerably reduces the level of Dnmt1 and, to less extent, Dnmt3a and Dnmt3b2/3 (isoform of Dnmt3b) MTases in T24 cells and others due to covalent attachment of the enzymes to d^{4H}Cyd-DNA. However, not all cell lines were sensitive to d^{4H}Cyd. Good results were obtained when tumor cells were sequentially treated with ZdCyd and d^{4H}Cyd. This led to deceleration of remethylation of the *p16* gene 5′-region and its blockage. It was supposed that specialized combination of these pharmaceuticals together with immunotherapy might me an effective treatment for cancer.

FdCyd compared with ZCyd, ZdCyd, and d^{4H}Cyd appeared to be a less promising drug because it undergoes enzymatic deamination to form toxic 5-fluorodeoxyuridine and its metabolites [74]. FdCyd demonstrates some advantages in experiments *in vitro*, since FdCyd and FdCyd-DNA are less sensitive to chemical hydrolysis.

An important problem in clinical application of MTase inhibitors is unspecific influence on healthy cells together with tumor cells. In connection with this, d^{4H}Cyd seems to be the most promising antitumor agent because it preferentially targets cancer cells in terms of incorporation into DNA, cell growth inhibition, demethylation of genes, and lowering the MTase expression level [72, 73].

Use of mechanism-based inhibitors for studies on structure and catalytic mechanism of MTases. *X-Ray structural analysis (XRSA).* Mechanism-based inhibitors are prime tools for studies on the structure and catalytic

mechanism of MTases. Four nucleoside inhibitors, namely FdCyd [10], 4′-SdCyd [30], DZdCyd [25], and d^{4H}Cyd [29] were used for structural analyses of M.HhaI–DNA complexes. Crystal structures of the ternary complexes of M.HhaI with 13-mer palindromic DNA duplex containing FdCyd instead of target Cyd in both strands and AdoMet (F13 structure, resolution 2.8 Å); with non-palindromic DNA duplex containing 4′-SdCyd instead of target dCyd in hemi-methylated M.HhaI recognition site and AdoMet (S13 structure, resolution 2.05 Å); with non-palindromic DNA duplex containing DZCyd instead of target dCyd in hemi-methylated recognition site and AdoHcy (DZ13 structure, resolution 2.55 Å); and with non-palindromic DNA duplex containing d^{4H}Cyd instead of target dCyd in one strand and AdoHcy (Z13 structure, resolution 2.5 Å) were analyzed.

In the case of F13 crystallization, M.HhaI–DNA conjugates were formed with covalent linkage between the Cys81 sulfur atom implicated in catalysis and the C6 carbon atom of 5-fluorocytosine and generation of 5-methyl-5-fluorodihydrocytosine and AdoHcy [10]. It was shown in this study that DNA is localized in a cleft between the large and small domains of the enzyme and maintains B-conformation except for the G·FC pair comprising 5-fluorocytosine instead of the target cytosine. This residue is swung completely out of the DNA helix and fitting snugly into the active-site pocket of the enzyme, which undergoes considerable conformational changes. DNA makes contacts with M.HhaI both by the major and minor grooves. Specific contacts are formed in the major groove with implication of two Gly-rich loops of the enzyme small domain. 5-Fluorocytosine, Cys81, and CH_3-donor AdoMet are spatially drawn together, which enables methylation. Thus, solution of the F13 structure led to a fundamental discovery: methylation implies flipping of the cytosine residue to be methylated. It became understood how the Cys residue of the MTase active site executes a nucleophilic attack on the target cytosine. The XRSA of the F13 structure is the first direct evidence for the supposed methylation mechanism and has demonstrated a novel type of sequence-specific recognition.

The S13 structure is similar to those of previously prepared ternary complexes composed of M.HhaI, DNA, and AdoHcy [10, 75, 76]. The flipping of a cytosine residue (from 4′-SdCyd) out of the DNA helix was observed. Surprisingly, the flipped cytosine residue in the S13 crystal structure (unlike a solution) was partially methylated. This resulted from a very slow reaction in the crystal, where dissociation of the enzyme–substrate complex is impossible. The reaction was facilitated by proper orientation of the target cytosine in the active site. Thus, the XRSA of the M.HhaI·(4′-SdCyd-DNA)·AdoMet complex first enabled direct visualization of the methyl group transfer from the donor atom (sulfur atom in AdoMet) to the acceptor atom (activated C5 carbon atom in the cytosine residue).

The DZ13 structure containing 5,6-dihydro-5-azacytosine shows that this target analog is also flipped out of the DNA double helix and occupies the catalytic pocket of the enzyme similarly to 5-fluorocytosine in the F13 structure. However, the distance between the C6 carbon atom and the sulfur atom of Cys81 (3.1 Å) exceeds the length of a covalent bond (1.8 Å), that is, a covalent bond is not formed between the sulfur atom of Cys81 sulfhydryl group and C6 carbon atom of DZdCyd [25]. The distance between Glu119 and the N3 nitrogen atom of DZdCyd, which should undergo protonation according to the reaction mechanism (Scheme 3a), is 3.3 Å compared to 2.8 Å in the F13 structure. This suggests the absence of protonation of the N3 nitrogen atom. An additional hydrogen bond is formed instead between the hydrogen atom at the N5 position and a water molecule, which results in stabilization of the complexes. All other contacts of M.HhaI with DZdCyd are the same as those in the F13 complex [10].

The Z13 complex closely resembles the F13 complex. The 2-pyrimidinone residue is flipped out of the DNA helix. The absence of an NH_2-group at the C4 position of the 2-pyrimidinone residue leads to the loss of two hydrogen bonds between the enzyme and DNA. M.HhaI formed a conjugate with d^{4H}Cyd-DNA. The distance between the C6 carbon atom of 2-pyrimidinone and the sulfur atom of Cys81 was 1.84 Å, corresponding to the length of a covalent bond (this distance was 2.6 Å in the complex with FdCyd). The distance between Glu119 and the N3 nitrogen atom, which should be protonated according to the methylation mechanism (Scheme 3a), was 2.91 Å (2.8 Å in the F13 structure) suggesting hydrogen bond formation. It is likely that protonation of the N3 nitrogen atom of target dCyd and attack by the sulfhydryl group on the C6 carbon atom occur coordinately.

Thus, crystal analyses of M.HhaI in complexes with DNA containing the target dCyd analogs FdCyd, 4′-SdCyd, DZdCyd, and d^{4H}Cyd have shown non-specificity to the target base during formation of complexes between M.HhaI (and possibly other MTases) with DNA. Solutions of these structures have made a major contribution to our understanding of the detailed mechanism of DNA methylation by MTases.

Interaction with mutant MTase forms. Studies on the mechanism-based inhibition of MTases and their mutant forms have revealed the amino acid residue implicated in nucleophilic attack on the C6 carbon atom of cytosine to be methylated (Scheme 3a). The main candidate for the role of nucleophile in MTases is the thiol group of a highly conserved Cys residue in the PCQ motif [1, 2] (Cys71 in M.HaeIII, Cys174 in M.MspI, Cys186 in M.EcoRII, etc.). To determine the role of Cys174 in M.MspI, a formation of conjugates between M.MspI, as well as its mutants C174S/T/Y, and d^{4H}Cyd-DNA was studied

(table) [28]. Only mutants C174S/T could form conjugates. N-Ethylmaleimide (NEM) selectively modifying SH-groups blocked formation of the conjugate M.MspI–(d^{4H}Cyd-DNA), but not C174S/T–(d^{4H}Cyd-DNA), because OH-groups of Ser and Thr are not modified by NEM.

The role of Cys186 in M.EcoRII was elucidated from studies on formation of conjugates between M.EcoRII or its mutants C186S/G/V/W [77-79] and ZCyd-DNA *in vitro* [79] and *in vivo* [77] or FdCyd-DNA [78] (table). The sensitivity of *E. coli* (*recA*$^-$) cells producing C186S/G/V/W to ZCyd was also examined [77]. The cells would die if the mutant MTase forms conjugates with ZCyd-DNA. The cells were only sensitive to ZCyd in the case of C186S. Hence, only C186S forms conjugates with ZCyd-DNA *in vivo*. In experiments *in vitro*, C186S formed conjugates with ZCyd-DNA [79], but not with FCyd-DNA [78]. Both the wild-type M.EcoRII and C186S mutant were treated with 5,5-dithio-bis(2-nitrobenzoic acid), a reagent modifying SH-groups in the same manner as does NEM. Virtually all activity of the wild-type enzyme disappeared, whereas the C186S mutant remained active.

Thus, the use of mechanism-based MTase inhibitors in combination with mutagenesis and treatment with selective reagents has allowed determination that the SH-groups of highly conserved Cys174 in M.MspI and Cys186 in M.EcoRII play a role of nucleophile during formation of covalent intermediate (Scheme 3a, II) in the methylation reaction. Formation of conjugates C174S/T–(d^{4H}Cyd-DNA) and C186S–(ZCyd-DNA) by M.MspI and M.EcoRII, respectively, is explained by elevated electrophilicity of the C6 position in the 2-pyrimidinone residue compared to that in the cytosine residue [29].

NON-NUCLEOSIDE INHIBITORS

Inhibition of MTases by nucleoside inhibitors is accompanied by formation of covalent MTase intermediates with DNA. This mechanism determines the high cytotoxicity of these inhibitors. So, it is necessary to search for substances that are only specific to MTases and do not affect DNA. Antisense oligonucleotides, oligonucleotide and DNA-duplex analogs of natural MTase substrates possessing higher affinity to MTases than the natural ones possess [31-33], as well as small interfering RNAs causing degradation of MTase mRNAs [80] have been tested. Another possibility is in searching for small molecules that specifically block MTases. Several low molecular weight substances that can inhibit activity of MTases in human cells have been described [81-83].

Of peculiar interest are MTase low molecular weight inhibitors isolated from natural sources (nutritional polyphenols), such as (–)-epigallocatechin-3-gallate (EGCG, Scheme 1c) from green tea [34] and isoflavones from soybean: genistein (4′,5,7-trihydroxyisoflavone) and its structural analogs biochanin A and daidzein [35]. Isoflavones are phytoestrogens.

Both EGCG and genistein can inhibit methyltransferase activity *in vitro* in nuclear extracts of esophageal cancer cell line KYSE 510 (IC_{50} = 20 and 67 μM, respectively) [34, 35]. It is known that some genes in KYSE 510 cells, such as the retinoic acid receptor-β (*RARβ*) gene, *p16*, and O6-methylguanine-DNA-methyltransferase (*MGMT*) gene, are inactive because of hypermethylation [34, 84]. Both EGCG and genistein caused demethylation of these genes *in vivo* and their reactivation. Effects of removal of hypermethylation and activation of genes after treatment with EGCG or genistein were also observed in other human tumor cell lines, for instance, in prostate cancer PC3 cells. Kinetic studies have demonstrated a competitive mechanism of inhibition by EGCG (K_i ~ 7 μM), whereas genistein acts by mixed (K_{ic} ~ 189.3 μM) and non-competitive (K_{in} ~ 80.2 μM) mechanisms.

Molecular modeling has shown that EGCG can be situated in the catalytic pocket of Dnmt1 and form hydrogen bonds with the enzyme, which are similar to those possibly stabilizing the target cytosine in the transient enzyme–substrate complex [34]. Biochanin A and daidzein appeared to be less effective DNA methylation inhibitors than genistein and EGCG [35]. The treatment of tumor cells with EGCG, genistein, biochanin A, or daidzein for several days revealed a dose-dependent inhibition of cell growth; biochanin A and daidzein less effectively inhibited the cell growth than did EGCG and genistein (biochanin A was the very poor inhibitor). Genistein had no effect on expression of genes encoding Dnmt1, Dnmt3a, and Dnmt3b MTases. Genistein activity in reactivation of genes *RARβ*, *p16*, and *MGMT* and in inhibition of cell growth increased when it was used in combination with trichostatin and sulforane (histone deacetylase inhibitors, the latter found in broccoli) or ZdCyd. Thus, among all tested substances EGCG from green tea and genistein from soybean are of interest as promising MTase inhibitors, whose putative mechanism of inhibition implicates their specific interaction with MTases. However, there is no evidence for direct inhibition of MTases by these substances. Their pharmacological effect on DNA methylation might be indirect [83].

Another promising class of MTase inhibitors comprises bisulfide bromotyrosine derivatives, psammaplins, isolated from the marine sponge *Pseudoceratina purpurea* (Scheme 1c) [27]. Psammaplins inhibit both MTases including Dnmt1 and histone deacetylases, thus exerting simultaneous effect on two epigenetic genome modifications. Novel members of this class, bisaprasin and psammaplins A and G, effectively inhibited both Dnmt1 and histone deacetylase (IC_{50} = 3.4-18.8 nM) with psammaplin G only demonstrating a small selectivity to

Dnmt1. Psammaplins also inhibit some other enzymes interacting with DNA, such as human topoisomerase II [81, 85]. As in the case of EGCG and genistein, there is no evidence for direct MTase inhibition by psammaplins. Psammaplins are offered as primary substances for further development of anticancer pharmaceuticals.

The 4-aminobenzoic acid derivatives procaine and procainamide (Scheme 1c) were approved by US Food and Drug Administration for application as local anesthetics and antiarrhythmics, respectively. They can bind to CpG-rich DNA sequences [40]. Initially, the inhibitory effect of procaine and procainamide on MTases was related to blocking the recognition or binding with CpG sites of DNA by MTases due to the binding with these sites. However, it was later shown that these drugs taken at micromolar concentrations inhibit Dnmt1 due to binding with it [41]. Their demethylating activity in tumor cells led to re-expression of tumor suppressor genes. Procaine was the primary substance for further modifications aimed at optimization of its therapeutic property [42]. To diminish the number of conformational states of the procaine molecule, the N-alkylamide fragment of the primary molecule was fixed in the form of 4- or 5-substituted oxazoline ring. The procaine analog (bottom part of Scheme 1c) had the most prominent inhibitory effect on the human myeloid leucosis HL60 cells. Treatment of the cells with this substance resulted in slight decrease of the total methylation level with no cytotoxic effect.

Among all non-nucleoside MTase inhibitors known to date, a substance of particular interest is RG108 (2-(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)-3-(1H-indol-3-yl)propanoic acid, Scheme 1c), whose molecule contains phthalimide and L-tryptophan residues [37, 83]. This substance was found using computer modeling of complexes between the catalytic domain of human Dnmt1 [86] and possible inhibitors acquired from the National Cancer Institute (USA) Low Molecular Weight Compounds (NCI/LMC) database. RG108 effectively blocks methyltransferase activity both *in vitro* (demonstrated on a model CpG-recognizing MTase M.SssI, IC_{50} = 115 nM) and *in vivo* in human tumor cells: incubation of colorectal cancer cells with micromolar concentrations of RG108 led to considerable demethylation of genomic DNA with no conspicuous cytotoxicity. The mechanism of inhibition by RG108 does not involve covalent linkage of enzyme with the inhibitor. An important role in interaction between RG108 and the active site of MTases belongs to the carboxyl group of the inhibitor, which is indicative of specific character of interaction between this substance and MTases [37, 83]. RG108 induced demethylation and reactivation of tumor suppressor genes and had no effect on the methylation status of centromeric satellite DNA. This is a very important advantage of RG108 over other demethylating drugs because chromosomal structure, which can be destabilized by hypomethylation of satellite DNA, remains unaffected [4, 5]. These data imply that RG108 is the MTase inhibitor with novel beneficial features. Thus, RG108 is a very promising primary substance for development of a novel class of MTase inhibitors whose design is targeted on the blocking of the active centers of these enzymes.

Chemotherapy with antibiotics capable of intercalation into DNA (amsacrine, actinomycin, mitoxantrone, and doxorubicin (Adriamycin)) is one of most successful methods of cancer therapy [87]. These intercalators have a common structural motif, a planar polycyclic aromatic system interposing between nucleotide pairs of DNA. The anthracyclin derivative doxorubicin is used in therapy of a wide spectrum of solid tumors of breast, urinary bladder, thyroid gland, lung, ovary, and stomach as well as osteosarcomas, lymphomas, and some leucoses [88]. Antitumor properties of doxorubicin are determined by inhibition of DNA-polymerase and topoisomerase (it forms a complex with topoisomerase II [89, 90], thus guiding tumor cells to apoptosis). For example, doxorubicin taken at a strict concentration caused apoptosis of human colorectal carcinoma HCT116 cells. It is likely that doxorubicin can also influence activity of other enzymes, particularly inhibiting Dnmt1 MTase [91]. It was shown that, as in the case of procainamide analogs, Dnmt1 is inhibited by doxorubicin *in vitro*. The mechanism of inhibition implies intercalation of doxorubicin into DNA, which affects the interaction of the enzyme with DNA. Dnmt1 is supposed to be one of the main doxorubicin targets that determines the doxorubicin-induced apoptosis of human tumor cells. Thus, doxorubicin is an inhibitor of MTases that indirectly influences their activity.

It should be stated in conclusion that hypermethylation of tumor suppressor genes is a characteristic feature of all malignant tumor types. This is equivalent to genomic DNA mutations leading to the loss of gene functions. The potential for correction of epigenetic mutations excites great interest in creation of MTase inhibitors. The best-known MTase inhibitor, 5-azacytidine, has been recently certified for therapeutic use and has performed well in medical care of patients with myelodysplastic syndrome. 5-Azacytidine, decitabine, zebularine, and other mechanism-based inhibitors are incorporated into genomic DNA. This determines their high cytotoxicity. A series of non-nucleoside substances decreasing the methylation level *in vivo* has been found, albeit, they usually cannot specifically inhibit MTases. So, further search is required for MTase inhibitors that are capable of specific interaction with these enzymes, in particular, with blocking of their active centers. During this search, it is necessary to estimate effects of potential MTase inhibitors on expression of genes, such as proto-oncogenes, genes associated with invasion and metastasis formation, imprinted genes, etc., whose activation via demethylation promotes tumor appearance and progression, as well as on the methylation status of repeated DNA sequences. It

is desirable in prospect to create pharmaceuticals enabling a directed turn-on/turn-off of certain genes: demethylation for their reactivation or, inversely, methylation for cessation of their transcription.

This study was supported by the Russian Foundation for Basic Research (grants 07-04-00583 and 08-04-01096) and RFBR/CRDF (grant 08-04-91109/RUB1-2919-MO-07).

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