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Inhibition of Murine DNA Methyltransferase Dnmt3a by DNA Duplexes Containing Pyrimidine-2(1*H*)-one

N. A. Cherepanova[1], A. L. Zhuze[2], and E. S. Gromova[1]*

[1]*Faculty of Chemistry, Lomonosov Moscow State University, 119991 Moscow, Russia; fax: (495) 939-3181; E-mail: gromova@genebee.msu.ru; cherepanova@genebee.msu.ru*
[2]*Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, ul. Vavilova 32, 119991 Moscow, Russia*

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Abstract—Here we studied the inhibition of the catalytic domain of Dnmt3a methyltransferase (Dnmt3a-CD) by DNA duplexes containing the mechanism-based inhibitor pyrimidine-2(1*H*)-one (**P**) instead of the target cytosine. It has been shown that conjugates of Dnmt3a-CD with **P**-DNA (DNA containing pyrimidine-2(1*H*)-one) are not stable to heating at 65°C in 0.1% SDS. The yield of covalent intermediate increases in the presence of the regulatory factor Dnmt3L. The importance of the DNA minor groove for covalent intermediate formation during the methylation reaction catalyzed by Dnmt3a-CD has been revealed. **P**-DNA was shown to inhibit Dnmt3a-CD; the IC_{50} is 830 nM. The competitive mechanism of inhibition of Dnmt3a-CD by **P**-DNA has been elucidated. It is suggested that therapeutic effect of zebularine could be achieved by inhibition of not only Dnmt1 but also Dnmt3a.

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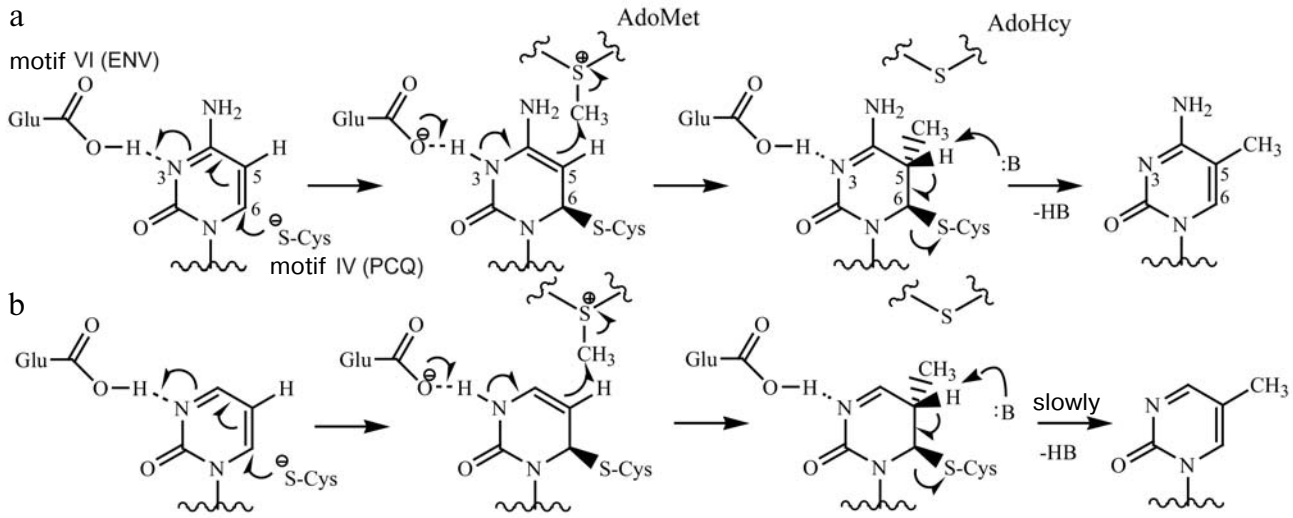
Key words: pyrimidine-2(1*H*)-one, covalent intermediate, competitive inhibition, eukaryotic DNA methyltransferase

Methylation of DNA is a natural epigenetic modification of the genome; it plays an important role in the regulation of many biological processes such as regulation of imprinting, transcription of genes, maintaining of chromatin structure, and inactivation of the X chromosome [1, 2]. DNA in eukaryotic cells is methylated by DNA methyltransferases (C5-MTases) of the Dnmt family (Dnmt1, Dnmt3a, and Dnmt3b) that methylate the C5 carbon atom of cytosine residues in CpG-sequences. C5-MTases use *S*-adenosyl-$_L$-methionine (AdoMet) as a methyl group donor, which turns into *S*-adenosyl-$_L$-homocysteine (AdoHcy) during the methylation reaction. Dnmt1 performs predominantly maintaining methylation, and MTases Dnmt3a and Dnmt3b perform *de novo* methylation [1, 3]. It has been shown that all three C5-MTases of the Dnmt family are important both for establishment and for maintenance of normal DNA methylation pattern [4], disturbance of which is observed in many tumors [5-7]. The DNA methylation reaction has been studied in detail for the prokaryotic C5-MTase HhaI (M.HhaI) [4] (Scheme). This process includes formation of C5-MTase/DNA/AdoMet ternary complex and subsequent flipping of the target cytosine from the DNA double helix, which is followed by movement of the catalytic loop towards the DNA minor groove. The most important stage of the catalytic mechanism is the formation of a covalent DNA–enzyme intermediate as a result of nucleophilic attack of the target cytosine C6 position by a highly conserved cysteine residue from C5-MTase motif IV (Scheme, panel (a)) [8]. Covalent intermediates of enzyme with DNA containing cytosine analogs instead of target cytosine, i.e. 5-fluorocytosine (FC), 5-azacytosine (AzaC), or pyrimidine-2(1*H*)-one (**P**), have been obtained for some prokaryotic C5-MTases [9, 10]. In this case FC-, AzaC-, and **P**-containing DNA (**P**-DNA) act as mechanism-based inhibitors of C5-MTases. The effect of this type of inhibitors is based on the fact that once formed, covalent intermediate either cannot be degraded or its degradation proceeds extremely slowly (Scheme, panel (b)).

Zebularine (1-β-D-ribofuranosyl-pyrimidine-2(1*H*)-one) is a promising candidate for development of antitu-

Abbreviations: AdoHcy, *S*-adenosyl-$_L$-homocysteine; AdoMet, *S*-adenosyl-$_L$-methionine; AzaC, 5-azacytosine; C5-MTase, C5-cytosine-DNA-methyltransferase; DB(11), dimeric bisbenzimidazole; FAM, 6-carboxyfluorescein; FC, 5-fluorocytosine; GST, glutathione S-transferase; NEM, N-ethylmaleimide; **P**, pyrimidine-2(1*H*)-one; *P*, fluorescence polarization; **P**-DNA, DNA containing pyrimidine-2(1*H*)-one; T_m, melting temperature.

* To whom correspondence should be addressed.



Mechanism of DNA methylation by C5-MTase M.HhaI; DNA contained cytosine in the target cytosine position (native DNA-substrate) (a) or pyrimidine-2(1*H*)-one (b) [10]

mor drugs among the variety of target cytosine analogs because its partial selectivity toward tumor cells *in vivo* has been shown. Incorporation of zebularine into DNA sequence caused inhibition of cell growth, demethylation of some gene promoters, and decrease in expression levels of C5-MTases in cells [10-14]. The mechanism of inhibitory effect of DNA containing 1-β-D-2′-deoxyribofuranosyl-pyrimidine-2(1*H*)-one instead of 2′-deoxycytidine was characterized on a variety of prokaryotic C5-MTases [15-17]. The **P** residue differs from cytosine by the lack of an exocyclic amino group that promotes inhibition of covalent intermediate dissociation. Covalent intermediate can be directly observed in the structure obtained for the complex of M.HhaI with **P**-DNA and AdoHcy [18]. Analogous covalent intermediates were separated and characterized for some prokaryotic C5-MTases: M.MspI [15], M.HgaI-2 [15], M.EcoRII [16], and M.SssI [17]. Data on inhibition of eukaryotic C5-MTases by **P**-DNA are limited and are related only to Dnmt1 [19]. It has been suggested that potential therapeutic effect of zebularine could be achieved by inhibition not only of Dnmt1 but also of other eukaryotic C5-MTases. In particular, the inhibition of a very important eukaryotic C5-MTase Dnmt3a by **P**-DNA has not been studied yet.

Dnmt3a can incorporate a methyl group into unmethylated DNA as well as into DNA already containing a methyl group in one of the strands (hemi-methylated DNA) [20]. Dnmt3a *in vitro* works on a distributive mechanism and methylates DNA at a low rate [21]. Naturally, Dnmt3a works in complex with the regulatory factor Dnmt3L [22, 23], which has been shown to stimulate catalytic activity of Dnmt3a from 3 to 20 times [24-27]. The heterotetrameric structure of the Dnmt3a catalytic domain (Dnmt3a-CD) and Dnmt3L complex consisting of two Dnmt3a-CD subunits and two Dnmt3L subunits (3L·3a·3a·3L) has been revealed [28]. It has been suggested that the stimulating effect could be achieved by restriction of the Dnmt3a catalytic loop conformational space by Dnmt3L in this complex (supporting of "closed" catalytic loop conformation), which allows the reaction to proceed more effectively [26, 28, 29].

It should be noted that the mechanism of interaction between Dnmt3a and DNA is poorly understood and there are some contradictory data about the participation of the conserved cysteine residue in this interaction [30, 31]. For instance, substitution of conserved Cys706 residue of C5-MTase Dnmt3a to alanine did not lead to the loss of catalytic activity [30]. The presence of ten conserved motifs typical for C5-MTases in the primary structure of Dnmt3a indicates that its mechanism of interaction with DNA is similar to that of M.HhaI [29, 32].

This work was aimed to study the inhibition of Dnmt3a by **P**-DNA and to estimate the parameters of this process. The molecular mechanism of **P**-DNA action on murine Dnmt3a, properties of covalent intermediate, participation of C5-MTase cysteine residues in its formation, and also role of the regulatory factor Dnmt3L in covalent intermediate Dnmt3a–DNA formation were studied. In this work, the Dnmt3a-CD, which retains catalytic activity in the absence of the N-terminal regulatory domain of this C5-MTase, was used [33].

MATERIALS AND METHODS

Reagents. AdoHcy from Sigma (USA) and [CH_3-^{3}H]AdoMet (15 Ci/mmol, 67 μM) from Amersham Biosciences (Great Britain) were used in this work.

Synthesis of dimeric bisbenzimidazole DB(11) was described previously [34]. Oligodeoxyribonucleotides containing 1-β-D-2′-desoxyribofuranosyl-pyrimidine-2(1*H*)-one were synthesized by S. N. Mikhailov (Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow) (see Table 1). Other oligodeoxyribonucleotides were synthesized by Sintol (Russia) and purified from polyacrylamide gel. Fluorescently labeled substrates containing the 6-carboxyfluorescein residue (FAM) were used for visualization of products in polyacrylamide gel and also for investigation of Dnmt3a-CD/DNA binding. FAM is joined with the oligodeoxyribonucleotides 5′-end with an alkylamino-linker containing six methylene groups. Con-centration of oligodeoxyribonucleotides was determined by UV absorbance at 260 nm.

The following buffers were used: 20 mM Hepes-NaOH, pH 7.5, 100 mM KCl, 1 mM EDTA, 0.2 mM dithiothreitol (DTT) (A), 10 mM Tris-HCl, pH 7.9, 50 mM NaCl, 1 mM DTT (B), 10 mM Tris-HCl, pH 7.9, 50 mM NaCl (C).

Proteins and enzymes. Isolation of recombinant Dnmt3a-CD and Dnmt3L was described previously [35]. For isolation of Dnmt3a-CD, *E. coli* BL21(DE3) cells were transformed with the plasmid pET28a containing the sequence encoding murine Dnmt3a-CD with N-terminal His_6 cluster. For isolation of Dnmt3L, pET41b plasmid containing the sequence encoding Dnmt3L with N-terminal His_6 cluster and C-terminal GST-tag was used. The proteins were purified to ~95% purity by affinity chromatography on Co^{2+}-containing TALON affinity resin (Clontech, USA). Purity of prepared protein samples was determined by electrophoresis in 12.5% SDS-polyacrylamide gel. Protein concentration was determined using standard Bradford assay and calculated for the protein monomeric form. M.HhaI (120 μM) was kindly provided by S. Klimasauskas (Institute of Biotechnology, Vilnius, Lithuania).

Determination of DNA melting temperatures. DNA duplex (1 μM) melting was studied in buffer C on a Hitachi 150-20 spectrophotometer (Japan). The sample was heated from 40 to 95°C at rate 0.5°C/min. Absorption changes were recorded at 260 nm. T_m (melting temperature) was determined from the maximum of the differential melting curve.

Dnmt3a-CD/Dnmt3L complex formation. Dnmt3a-CD (4 μM) and Dnmt3L (1.2 μM) were mixed (the molar ratio was 1 : 1) in buffer A to final complex concentration of 600 nM (per protein monomer). The mixture was incubated at 37°C for 30 min. Time of incubation was chosen according to previous study [27].

Analysis of C5-MTase–P-DNA conjugates. The reaction mixtures contained 200 nM FAM-labeled duplex, 0.1 mM AdoMet or AdoHcy, 2 μM Dnmt3a-CD, or 300 nM Dnmt3a-CD/Dnmt3L or 1 μM M.HhaI. For reaction mixtures containing Dnmt3a-CD or Dnmt3a-CD/Dnmt3L buffer A was used, and for mixtures with M.HhaI buffer B was used. After incubation at 4°C for 60 min and at room temperature for 10 min SDS was added to the reaction mixture up to 1% concentration. Then half of the mixture was heated at 65°C for 5 min. In experiments with N-ethylmaleimide (NEM), C5-MTases were preincubated with 5 mM NEM for 10 min at room temperature and after that were added to the reaction mixtures. In experiments with DB(11), duplex f**MG**/G**P** (Table 2) was incubated for 3 days with DB(11) and then added to the reaction mixture. Both heated and unheated samples were applied on 12.5% polyacrylamide gel (Laemmli method) containing 0.1% SDS and analyzed by electrophoresis. Gels were imaged on a FUJIFILM FLA-3000 device (Japan). Conjugate yield was estimated with the Image Quant 5.2 program as the ratio of fluorescence intensity of conjugate band to the total fluorescence intensity of both bound and unbound DNA duplex.

Table 1. Oligodeoxyribonucleotide sequences

Designation	Sequence
CG	5'-GAGCCAAGCGCACTCTGA
PG	5'-GAGCCAAG**P**GCACTCTGA
CG**P**	5'-GAGCCAAGCG**P**ACTCTGA
MG	5'-GAGCCAAGMGCACTCTGA
fMG	5'-FAM-GAGCCAAGMGCACTCTGA
GM	3'-CTCGGTTCGMGTGAGACT
GMf	3'-CTCGGTTCGMGTGAGACT-FAM
G**P**	3'-CTCGGTTCG**P**GTGAGACT
30CG	5'-CTGAATACTACTTGCGCTCTCTAACCTGAT
30GC	5'-ATCAGGTTAGAGAGCGCAAGTAGTATTCAG

Note: M, 5-methylcytosine; **P**, pyrimidine-2(1*H*)-one; f, FAM (6-carboxyfluorescein).

DNA binding by Dnmt3a-CD. Binding of Dnmt3a-CD to DNA was studied using the fluorescence polarization technique with direct titration of FAM-labeled oligodeoxyribonucleotide duplexes by C5-MTase Dnmt3a-CD as described earlier [35]. Fluorescence polarization value (P) was calculated according to the equation $P = (I_v - GI_h)/(I_v + I_h)$, where I_v and I_h are vertical and horizontal components of emitted light, respectively, and G is a correction factor. DNA duplex (10 nM) containing FAM-label in one of the strands was incubated with 0.1 mM AdoHcy, and fluorescence polarization of the DNA duplex was estimated in the absence of enzyme (P_0). Then aliquots of Dnmt3a-CD solution were added and fluorescence polarization value was recorded 2 min after each addition. Measurements were performed using a Cary Eclipse spectrofluorimeter (Varian, USA). The dependence of P value versus Dnmt3a-CD concen-

Table 2. Melting temperature of **P**-DNA and dissociation constants of complexes of Dnmt3a-CD with **P**-DNA

DNA duplex*	Designation*	T_m (±1), deg	K_d, nM***
5'- GAGCCAAG**C**<u>**G**</u>CACTCTGA 3'- CTCGGTTC**G**<u>**M**</u>GTGAGACT	**C**<u>**G**</u>**/GM**	67.0	214 ± 20
5'- GAGCCAAG<u>**P**</u>**G**CACTCTGA 3'- CTCGGTTC**G**<u>**M**</u>GTGAGACT	<u>**P**</u>**G/GM**	42.5** 57.4	143 ± 16
5'- GAGCCAAG**C**<u>**G**</u>PACTCTGA 3'- CTCGGTTC**G**<u>**M**</u>GTGAGACT	**C**<u>**G**</u>**P/GM**	56.7	185 ± 20
5'- GAGCCAAG**MG**CACTCTGA 3'- CTCGGTTC**G**<u>**P**</u>GTGAGACT	**MG/G**<u>**P**</u>	56.8	120 ± 14

* Designations of oligodeoxyribonucleotides are the same as in Table 1. Recognition site for Dnmt3a-CD is in bold, cytosine to be methylated or its substitution **P** is underlined.

** T_m is shown for every step of biphasic melting curve.

*** Dissociation constants of complexes of Dnmt3a-CD with FAM-labeled **P**-DNA in the presence of AdoHcy.

tration was plotted. Curves of titration of each DNA duplex by C5-MTase were reproduced at least in triplicate and were analyzed with the Origin 7.5 program according to Hill equation:

$$\frac{(P - P_0)}{(P_m - P_0)} = \frac{[E]^n}{[E]^n + [K_d]^n},$$

where P_0 and P_m are fluorescence polarization values of free and completely bound FAM-labeled DNA, [E] is Dnmt3a-CD concentration, and n is the Hill coefficient.

Methylation of DNA by Dnmt3a-CD/Dnmt3L complex in the presence of P-DNA. DNA methylation percent was determined according to incorporation of tritium label from [CH_3-^{3}H]AdoMet to DNA during the methylation reaction. Reactions proceeded for 30 min at 37°C in 10 μl of buffer A. The reactions were initiated by adding the enzyme. Reaction mixtures were applied on DE81 ion-exchange filters (Whatman, Great Britain) as described earlier [16], and concentration of transferred ^{3}H-methyl groups was calculated as described in [36].

Determination of IC_{50}. Reaction mixtures contained 0.5 μM DNA duplex 30**C**<u>**G**</u>/30**G**<u>**C**</u>, 50 nM Dnmt3a-CD/Dnmt3L, 1.3 μM [CH_3-^{3}H]AdoMet, and 0-3 μM inhibitor <u>**P**</u>**G/GM** or **MG/G**<u>**P**</u>. The dependence of methylation percent versus inhibitor concentration was plotted. Values of IC_{50} were estimated as concentrations of inhibitors that caused 50% decrease in the methylation reaction yield.

Determination of K_i. Reaction mixtures containing 150-1500 nM DNA duplex 30**C**<u>**G**</u>/30**G**<u>**C**</u>, 150 nM Dnmt3a-CD/Dnmt3L, and 2 μM [CH_3-^{3}H]AdoMet were incubated in the presence of 0-200 nM DNA duplex **MG/G**<u>**P**</u>. Concentration of transferred methyl groups was calculated as described above. According to this data, initial rates of the methylation reaction (V_0) were determined. V_0 were plotted against substrate concentration and were subjected to nonlinear regression analysis using the Origin 8.0 program. Michaelis constant (K_m) and maximum rate (V_{max}) were obtained from data fitting. Then these values were linearized in double reciprocal coordinates to obtain K_i.

RESULTS AND DISCUSSION

Covalent intermediate formation during the methylation reaction. In order to examine the inhibition of Dnmt3a by **P**-DNA, we should first clarify if Dnmt3a forms covalent intermediates with **P**-DNA and study their properties. Each 18-mer oligodeoxynucleotide duplex (Table 2) contained overlapping recognition sites of control C5-MTase HhaI (5′-GCGC/3′-CGCG) and Dnmt3a-CD (CpG) and, thus, was used as a substrate for both MTases (Tables 2 and 3). The **P** residue was incorporated instead of the target cytosine in the upper or bottom strand (<u>**P**</u>**G/GM** and **MG/G**<u>**P**</u>, respectively) and also near the recognition site (**C**<u>**G**</u>**P/GM**) (Table 2). This allowed us to specify whether covalent intermediate of the methylation reaction forms and to examine the role of CpG-flanking sequences for Dnmt3a-CD functioning at the covalent intermediate formation stage.

To investigate the effect of cytosine replacement with **P** on DNA duplex stability, UV-melting curves were obtained and melting temperatures of DNA duplexes were determined (Table 2). Such modification of DNA decreased double helix stability in all cases. Destabilizing effects in the case of duplexes **C**<u>**G**</u>**P/GM** and **MG/G**<u>**P**</u> were equal to 10°C (Table 2). A biphasic UV-melting curve was observed for duplex <u>**P**</u>**G/GM**; the melting temperature of the first step was 42.5°C, i.e. the destabilizing effect was 24.5°C. Only two hydrogen bonds are possible

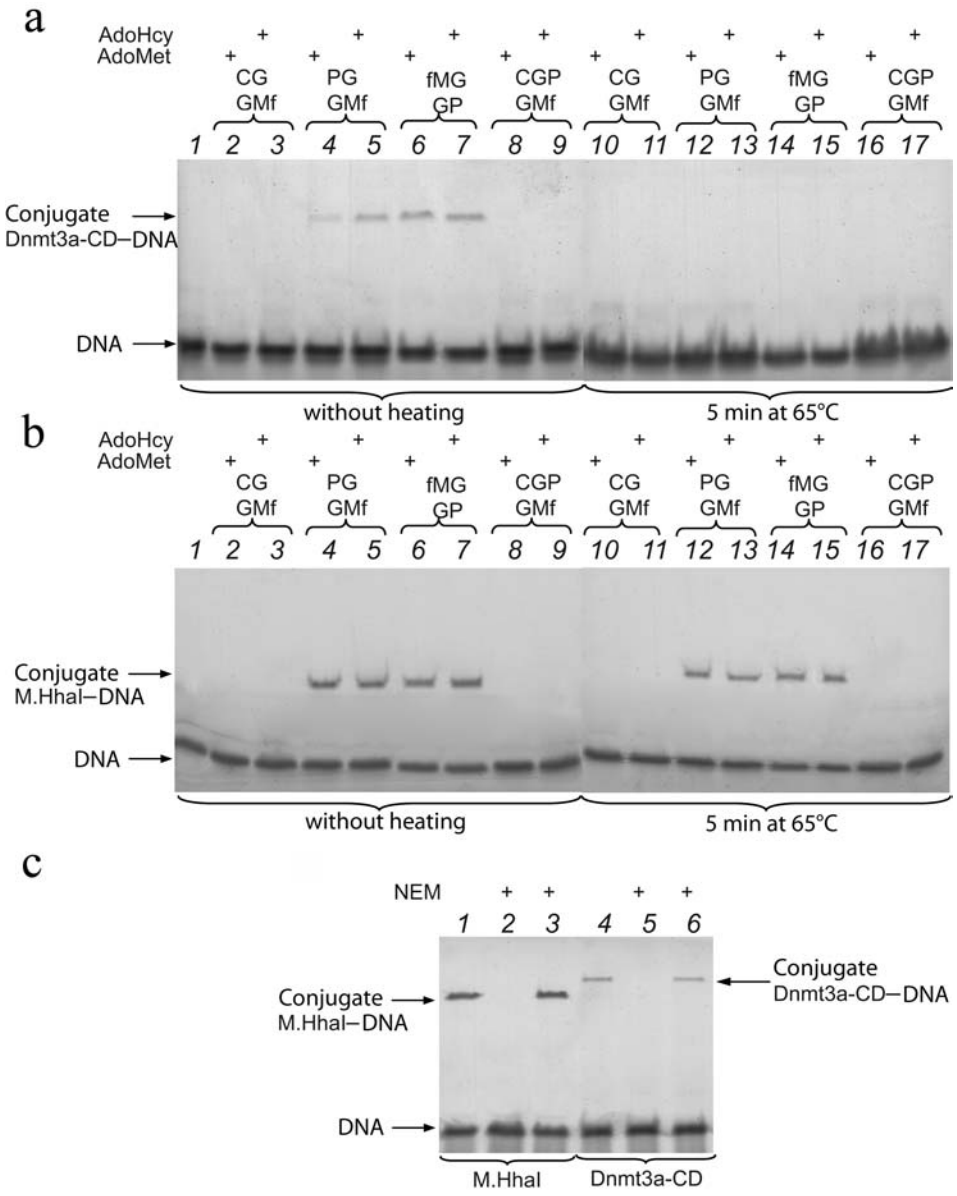


Fig. 1. Analysis of conjugate formation of Dnmt3a (a) and M.HhaI (b) with **P**-containing DNA. Reaction mixtures contained 2 μM Dnmt3a-CD (or M.HhaI), 200 nM corresponding DNA-duplex (**CG**/**GMf** for lanes *2*, *3*, *10*, *11*; **PG**/**GMf** for lanes *4*, *5*, *12*, *13*; f**MG**/**GP** for lanes *6*, *7*, *14*, *15*; **CGP**/**GMf** for lanes *8*, *9*, *16*, *17*), 0.1 mM AdoMet (lanes *2*, *4*, *6*, *8*, *10*, *12*, *14*, and *16*) or AdoHcy (lanes *3*, *5*, *7*, *9*, *11*, *13*, *15*, and *17*) in buffer A or B. Lane *1*: DNA-duplex **CG**/**GMf** alone. Reaction mixtures were incubated with 1% SDS for 10 min at room temperature (lanes *1-9*) or at 65°C (lanes *10-17*). c) Analysis of conjugate formation of M.HhaI and Dnmt3a-CD with DNA duplex f**MG**/**GP** in the presence of NEM (cysteine modification reagent). Concentrations of all reagents and C5-MTases are the same as for (a) and (b). Lanes: *1*, *4*) formation of conjugates of C5-MTases with 200 nM duplex f**MG**/**GP** in the presence of 0.1 mM AdoHcy; *2*, *5*) C5-MTases were preincubated with 5 mM NEM; *3*, *6*) NEM was added in reaction mixture after incubation with DNA. Fluorogram of 12% denaturing Laemmli polyacrylamide gel containing 0.1% SDS.

between guanine and **P** residues (one less than in ordinary GC-pair) [37]. However, this significant destabilizing effect cannot be unambiguously explained by loss of only one hydrogen bond in the G**P**-pair. Significant destabilizing effect of DNA duplexes containing **P** residue and its dependence on nucleotide context was observed earlier [16, 38]. Destabilizing effects might be so dramatic because of unfavorable electrostatic coupling caused by changed charge distribution in the **P** residue compared with a cytosine residue, which critically affects stacking interactions with neighboring base pairs. The presence of unfavorable electrostatic coupling of **P** with neighboring base pairs in DNA duplex was confirmed by molecular dynamics analysis [39].

The ability of Dnmt3a-CD to form covalent intermediate with FAM-labeled **P**-containing DNA duplexes

Table 3. The yields of conjugates of C5-MTases with **P**-DNA and inhibitory effects of **P**-DNA

DNA duplex*	Yield of conjugate of C5-MTase with **P**-DNA, %**				IC_{50}, μM***
	M.HhaI		Dnmt3a-CD		
	AdoMet	AdoHcy	AdoMet	AdoHcy	
CG/GM	–	–	–	–	
PG/GM	32 ± 7	30 ± 6	3.5 ± 1	7 ± 2	1.55 ± 0.26
CGP/GM	–	–	–	–	
MG/G**P**	35 ± 6	36 ± 8	10 ± 3	11 ± 3	0.83 ± 0.17

* Designation of duplexes is the same as in Table 2.

** Maximum yields were obtained according to Laemmli in 12.5% denaturing polyacrylamide gel containing 0.1% SDS with pretreatment of the samples with 1% SDS. $C_{M.HhaI}$ was 1 μM, $C_{Dnmt3a\text{-}CD}$ was 2 μM, C_{DNA} was 200 nM. FAM-labeled **P**-DNAs were used.

*** IC_{50} were determined for Dnmt3a-CD/Dnmt3L complex.

was then studied in polyacrylamide gel containing 0.1% SDS. Prokaryotic M.HhaI was used as a control enzyme. Detectable amounts of FAM-labeled material with reduced mobility in the gel were observed for both enzymes only in case of DNA substrates containing a **P** residue instead of the target cytosine (Fig. 1, a and b, lanes *4-7*) in the presence of both AdoMet and AdoHcy.

The stability of DNA–protein complexes during analysis at 25°C in 0.1% SDS Laemmli polyacrylamide gel suggests the existence of a covalent **P**-DNA–enzyme bond. However, covalent intermediates of both C5-MTases with parental DNA duplex **C**G/GMf are unstable and disrupt during gel-electrophoresis analysis (Fig. 1, a and b, lanes *2* and *3*). The presence of noncovalent complexes of Dnmt3a and M.HhaI with DNA can be excluded because both for parental duplex **C**G/GMf and for duplex **C**GP/GMf containing **P** near the CpG-site there are no low mobility products in the gel (Fig. 1, a and b, lanes *2* and *3*, *8* and *9*). Both Dnmt3a-CD and M.HhaI, being preincubated with cysteine-modifying reagent NEM, do not form conjugate with duplex fMG/G**P** (Fig. 1c, lanes *2* and *5*). Addition of NEM to the reaction mixtures after incubation of enzymes with DNA does not affect on the conjugate formation (Fig. 1c, lanes *3* and *6*). This fact again confirms that the formed enzyme–DNA link is covalent and does not disrupt under the influence of NEM. This means that formation of Dnmt3a–DNA conjugate requires the presence of the reactive cysteine residue in Dnmt3a. This cysteine residue is likely the highly conserved residue Cys706 from motif IV.

The yield of Dnmt3a–fMG/G**P** conjugate was higher than Dnmt3a–**P**G/GMf conjugate (Table 3). It is known that Dnmt3a methylates a CpG-site more effectively if its flanking sequence contains PyrPurCGPyr (where Pyr and Pur are pyrimidine and purine bases, respectively) [31]. The nucleotide context of **P**G-site in the case of **P**G/GMf was 5′-AG**P**GC and in case of fMG/G**P** was 5′-TG**P**GC (Table 2). The fMG/G**P** nucleotide context is more consistent with the preferable one for methylation of Dnmt3a. This can explain higher yield of conjugate in the case of fMG/G**P**.

It was also found that formed Dnmt3a-CD–**P**-DNA conjugates are not stable to heating at 65°C, in contrast to corresponding M.HhaI–**P**-DNA conjugates (Fig. 1, a and b, lanes *10-17*). Covalent intermediates of FC-containing substrate analogs with Dnmt3a-CD and M.HhaI remain resistant to 1% SDS when heated to 95°C [30, 40]. The inhibitory effect of FC is due to the presence of a fluorine atom instead of hydrogen atom in the C5 position, which cannot be deleted as a proton during the reaction. The inhibition of C5-MTases by **P**-containing substrates is based on increased reactivity of **P** residue C6 position, decreased energy barrier for base flipping, and retardation of proton β-elimination from the C5 position [18]. **P**-containing DNA duplexes do not cause irreversible enzyme inactivation. Reversible inhibition and lability of the covalent bond in the case of **P** might explain lower cytotoxity of zebularine in contrast to 5-azacytidine [19].

It is known that conjugates of M.HhaI, Dnmt1 [19], and M.EcoRII [16] with **P**-DNA also degrade when heated to 80°C. Conjugates of CpG-recognizing prokaryotic C5-MTase SssI with **P**-DNA cannot sustain heating even to 65°C [17]. Thus, thermal stability of conjugates of C5-MTases with **P**-DNA in the presence of SDS can be presented in the following order: M.HhaI > M.EcoRII > Dnmt1 > M.SssI ~ Dnmt3a.

It should be noted that the amino acid residue taking part in deprotonation of the cytosine C5 position in covalent intermediate C5-MTase–DNA remains unknown (Scheme). Differences in thermal stabilities of previously mentioned covalent intermediates C5-MTases–**P**-DNA can be explained by different amino acid content of

MTase active sites. It can be suggested that in the case of CpG-recognizing C5-MTases the amino acid residue responsible for the deprotonation of C5 position has more basic properties than the analogous residue in M.HhaI, resulted in facilitation of the C5 position deprotonation and conjugate dissociation.

Binding of Dnmt3a-CD to P-containing DNA duplexes. Low yield of Dnmt3a-CD–**P**-DNA conjugates (Table 3) might be related to the change in binding affinity of Dnmt3a-CD to modified DNA in comparison with parental DNA. To test this, binding of Dnmt3a-CD to FAM-labeled **P**-DNA duplexes was studied by fluorescence polarization assay in the presence of AdoHcy. AdoHcy being the analog of cofactor AdoMet promotes the formation of specific C5-MTase–DNA complex [41].

It should be noted that Dnmt3a presents in solution in its multimeric state [27] and forms nucleoprotein filaments with DNA [42]. Cooperative binding of Dnmt3a-CD to 30-mer DNA containing 7,8-dihydro-8-oxoguanine [35] and O6-methylguanine [43] was detected earlier. Binding curves were sigmoid in the case of all 18-mer DNA substrates (Fig. 2), whereas the plotted data had a reasonably good fit with the Hill equation (see "Materials and Methods"). Estimated values of dissociation constants (K_d) for ternary complexes of Dnmt3a-CD with duplexes **C**G/**GM**f, **C**GP/**GM**f, and **P**G/**GM**f and AdoHcy were similar (Table 2). A small decrease in K_d was observed in the case of duplex f**MG**/G**P**. Enhancement of binding affinity to **P**-DNA was observed earlier for methyltransferase MspI [44], which was explained by DNA–enzyme covalent binding. Hence, the affinity of Dnmt3a-CD to **P**-containing substrates **C**GP/**GM**f, f**MG**/G**P**, and **P**G/**GM**f is not decreased in comparison with this for parental substrate **C**G/**GM**f. This means that reduced Dnmt3a-CD–**P**-DNA conjugate yields in comparison to analogous conjugate yields of M.HhaI are not caused by cytosine to **P** substitution. Reduced conjugate yields might be a specific feature of Dnmt3a–DNA interaction; besides, it correlates with low methylating activity of Dnmt3a *in vitro* (see below).

Interaction between Dnmt3a-CD catalytic loop and DNA minor groove during the covalent intermediate formation. Further, the role of DNA minor groove in the catalytic mechanism of Dnmt3a-CD (during formation of covalent intermediate of the reaction) was studied. It was shown that formation of catalytically competent M.HhaI–DNA complex is accompanied by the contact formation of the enzyme catalytic loop with the DNA minor groove [45]. The similarity of M.HhaI and Dnmt3a primary structures [32] suggests that this conclusion might also be correct for Dnmt3a.

To test this suggestion, the covalent intermediate formation was studied in the case when DNA minor groove is occupied by DNA-binding ligand. Recently it was found that the presence of bulky benzo[*a*]pyrene residue in the DNA minor groove leads to a significant decrease

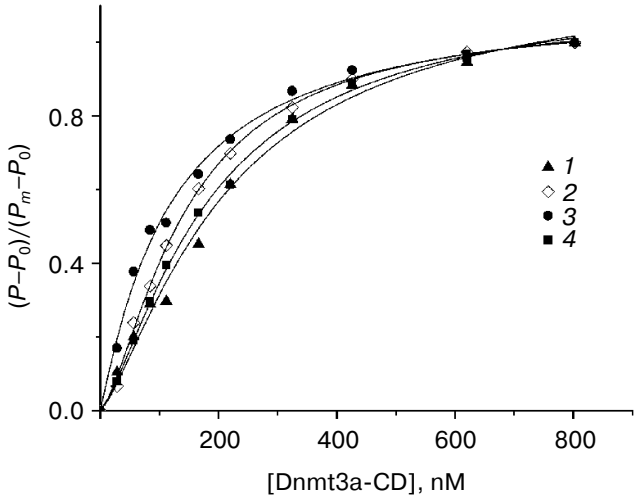
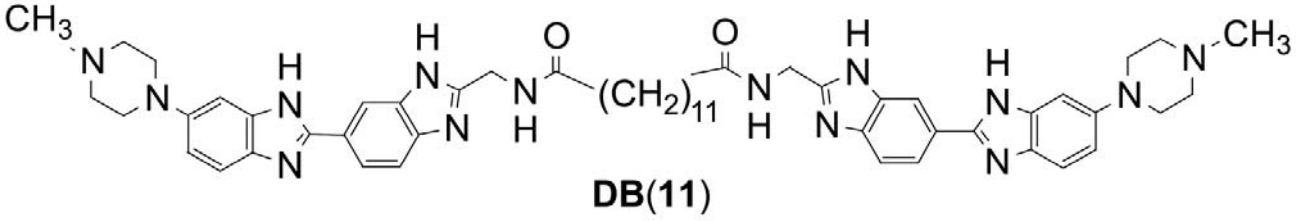


Fig. 2. Curves representing binding of 10 nM fluorescently labeled DNA duplexes (Table 2) to Dnmt3a-CD in the presence of 0.1 mM AdoHcy. P, fluorescence polarization; P_0 and P_m are fluorescence polarization values of free and completely bound FAM-labeled DNA. Labels: *1*) **C**G/**GM**f; *2*) **P**G/**GM**f; *3*) f**MG**/G**P**; *4*) **C**GP/**GM**f.

in catalytic activity of prokaryotic C5-MTases SssI and HhaI due to distortion of the enzyme catalytic loop contacts with the minor groove [46, 47]. Hoechst 33258 dye and its analogs are ligands that effectively bind to minor groove of AT-containing DNA and less effectively to heterogeneous DNA sequences [48]. Dimeric bisbenzimidazole DB(11) (Fig. 3a) is an analog of Hoechst 33258 and is also a minor groove-binding ligand [49]. DB(11) inhibits methylating activity of Dnmt3a-CD at low micromolar concentrations [34]. In the presence of increasing DB(11) concentrations, yield of covalent intermediate of Dnmt3a-CD with duplex f**MG**/G**P** decreases and then the corresponding band in gel completely disappears (Fig. 3b). Thereby, binding of DB(11) to DNA prevents the covalent intermediate formation during the methylation reaction. Dependence of covalent intermediate formation on the DB(11) presence in the DNA minor groove confirms the suggestion that Dnmt3a-CD and M.HhaI have similar catalytic mechanisms. The observed effect can be explained by distortion of contacts between the Dnmt3a-CD catalytic loop and groups of atoms exposed in the DNA minor grove as well as by disturbance of target cytosine flipping out of the DNA double helix. So the DNA minor groove is important for the Dnmt3a–DNA covalent intermediate formation in the methylation reaction.

Dnmt3L effect on the formation of covalent intermediate in the methylation reaction. It was shown that the interaction of the regulatory factor Dnmt3L with Dnmt3a lead to the formation of 3L·3a·3a·3L complexes, which possess enhanced catalytic activity in contrast to Dnmt3a [27]. Taking into account the stimulation of Dnmt3a catalytic

a



b

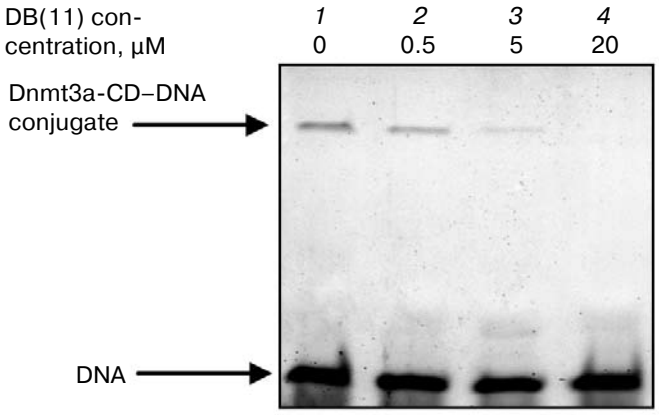


Fig. 3. Conjugate formation of Dnmt3a-CD with f**MG**/**G**<u>**P**</u> in the presence of DB(11). a) Structural formula of minor groove binding ligand DB(11); b) gel illustrating the formation of Dnmt3a-CD conjugate with duplex f**MG**/**G**<u>**P**</u> in the presence of an increasing concentration of DB(11) (0-20 μM) and 0.1 mM AdoHcy. Fluorogram of 12% denaturing Laemmli polyacrylamide gel containing 0.1% SDS.

activity by Dnmt3L, we studied the effect of Dnmt3L on Dnmt3a-CD–**P**-DNA covalent intermediate formation.

The Dnmt3a-CD/Dnmt3L complex forms covalent intermediates with DNA duplex f**MG**/**G**<u>**P**</u> in the presence of AdoMet or AdoHcy (Fig. 4a, lanes *2* and *3*). These conjugates degrade when heated to 65°C (Fig. 4a, lanes *4* and *5*) as well as corresponding conjugates formed in the absence of Dnmt3L (Fig. 1a, lanes *14* and *15*). Therefore, Dnmt3L does not increase the stability of the Dnmt3a–DNA covalent intermediate. However, Dnmt3L addition increases the yield of covalent intermediate. For instance, the yield of conjugate in the presence of 300 nM Dnmt3a-CD/Dnmt3L complex (9%) more than three times exceeds the yield of analogous conjugate with 300 nM Dnmt3a-CD in the absence of Dnmt3L (2.5%) (Fig. 4b).

It should be noted that conjugate yields of Dnmt3a-CD with **P**-DNA were significantly less than that for prokaryotic M.HhaI (Table 3) or M.SssI (30%) [17]. Catalytic constants of the methylation of duplex **<u>C</u>G**/**GM** by M.HhaI, M.SssI, and Dnmt3a-CD are 3.7 min^{-1}, 2.3 min^{-1} [46], and 3.0 h^{-1}, respectively (O. V. Lukashevich, M. V. Darii, and E. S. Gromova, unpublished data). So the yields of covalent intermediates of these C5-MTases correlate with methylation efficiency.

Taking into account the low yield of Dnmt3a-CD–**P**-DNA conjugate and low DNA methylation efficiency, one can suggest that in Dnmt3a-CD/DNA complex the catalytic cysteine of Dnmt3a-CD can be oriented in not an ideal conformation for nucleophilic attack of the target cytosine. This possibility was taken into consideration in the interpretation of the role of covalent catalysis in the catalytic mechanism of Dnmt3a [30]. Increasing of the covalent intermediate yield and, as a consequence, increasing of methylation efficiency can be explained by the fact that Dnmt3L binding to Dnmt3a promotes the transition of the catalytic loop into "closed" conformation that is optimal for catalysis [28].

P-DNA as Dnmt3a-CD/Dnmt3L inhibitors. The inhibitory potential of **P**-containing DNA towards Dnmt3a-CD caused by the formation of stable C5-MTase–DNA conjugates. It was necessary to estimate the parameters of Dnmt3a-CD inhibition by **P**-containing DNA. The ability of DNA duplexes **MG**/**G**<u>**P**</u> and <u>**P**</u>**G**/**GM** containing a **P** residue instead of the target cytosine to inhibit C5-MTase Dnmt3a-CD in complex with Dnmt3L was studied *in vitro*. Unmethylated 30-mer DNA duplex 30<u>**C**</u>**G**/30**G**<u>**C**</u>

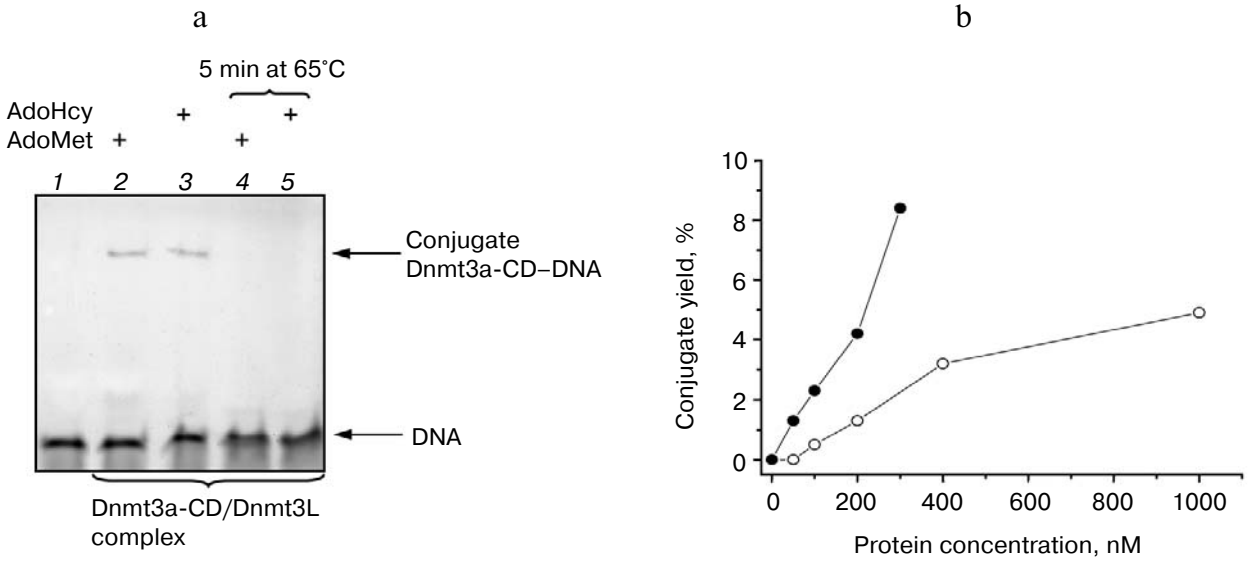


Fig. 4. a) Analysis of conjugate formation of Dnmt3a-CD/Dnmt3L (300 nM) with DNA-duplex f**MG/GP** (200 nM) in the presence of 0.1 mM AdoMet (lanes *2* and *4*) or AdoHcy (lanes *3* and *5*). Reaction mixtures were incubated with 1% SDS for 10 min at room temperature (lane *1-3*) or at 65°C (lanes *4* and *5*). Lane *1*: DNA-duplex f**MG/GP** alone. Fluorogram of 12% denaturing Laemmli polyacrylamide gel containing 0.1% SDS. b) Relation of the conjugates yield of Dnmt3a-CD (open circles) and Dnmt3a-CD/Dnmt3L (closed circles) with DNA duplex f**MG/GP** (200 nM) in the presence of 0.1 mM AdoHcy to enzyme concentration. Enzyme concentrations varied within the range 0-1000 nM for Dnmt3a-CD and 0-300 nM for Dnmt3a-CD/Dnmt3L.

5′-CTGAATACTACTTG**CG**CTCTCTAACCTGAT
3′-GACTTATGATGAAC**GC**GAGAGATTGGACTA

was used as an analog of native DNA-substrate of C5-MTase. The relations of 30**CG**/30**GC** methylation to inhibitor concentration were measured and IC_{50} values were determined (Table 3 and Fig. 5a). DNA duplexes **MG/GP** and **PG/GM** act as inhibitors of the methylation reaction at micromolar concentrations. Furthermore, DNA duplex **MG/GP** inhibits methylating activity of Dnmt3a-CD/Dnmt3L more effectively than **PG/GM**. This correlates with higher conjugate yield of Dnmt3a-CD with f**MG/GP** than that for analogous conjugate with **PG/GM**f (Table 3) because of nucleotide context near the CpG-site is more preferable for Dnmt3a-CD (see above).

It should be noted that there are rather few data about Dnmt3a inhibitors and about parameters of inhibition [9, 10]. However, we can say that nucleotide inhibitors studied in this work are more effective towards Dnmt3a (IC_{50} is 830 nM) than non-nucleotide inhibitors such as dimeric bisbenzimidazoles (IC_{50} was 5 μM [34]). At the same time, the inhibitory effect of the described **P-**DNAs towards Dnmt3a was lower than the inhibitory effects observed in the case of other **P-**DNAs towards Dnmt1 (IC_{50} were 160-240 nM [19, 50]).

IC_{50} values are useful for comparison of the inhibitory properties of different compounds, but they provide no information about the mechanism of their action. To determine inhibitory mechanism, DNA methylation by complex Dnmt3a-CD/Dnmt3L was studied in the presence of various concentrations of inhibitor **MG/GP**. The kinetic parameters of the methylation reaction were determined by the non-linear regression analysis using the Michaelis–Menten model (Fig. 5, b and c) from the dependence of initial methylation rates on substrate concentration. The Michaelis constant determined for 30**CG**/30**GC** was 390 ± 20 nM, which correlates with the previously obtained 250 nM Michaelis constant value for Dnmt3a-CD/Dnmt3L in reaction with another 30-mer DNA substrate [26]. Addition of the inhibitor increased the Michaelis constant; but the maximum rate of the reaction remained practically unchanged (Fig. 5b). Linearization of kinetic data in Lineweaver–Burk coordinates shows that the intersection of the lines lies on the ordinate axis (Fig. 5c). This indicates the competitive mechanism of Dnmt3a-CD/Dnmt3L inhibition by DNA duplex **MG/GP**. The inhibition constant was found to be 240 ± 30 nM, which correlates with dissociation constants determined in a direct way in the process of binding of **P-**DNA to Dnmt3a-CD (Table 2).

It is now clear that C5-MTase Dnmt3a plays an important role in different types of cells. The role of Dnmt3a functioning in carcinogenesis of such diseases as melanoma and colon cancer has been reported [51, 52]. The data of the present work suggest that oligonucleotide duplexes **MG/GP** and **PG/GM** are inhibitors of C5-MTase Dnmt3a-CD *in vitro*, and antitumor effect of zebularine could be achieved not only by inhibition of Dnmt1, but also by inhibition of Dnmt3a. The determined parameters of Dnmt3a-CD inhibition by **P-**DNA

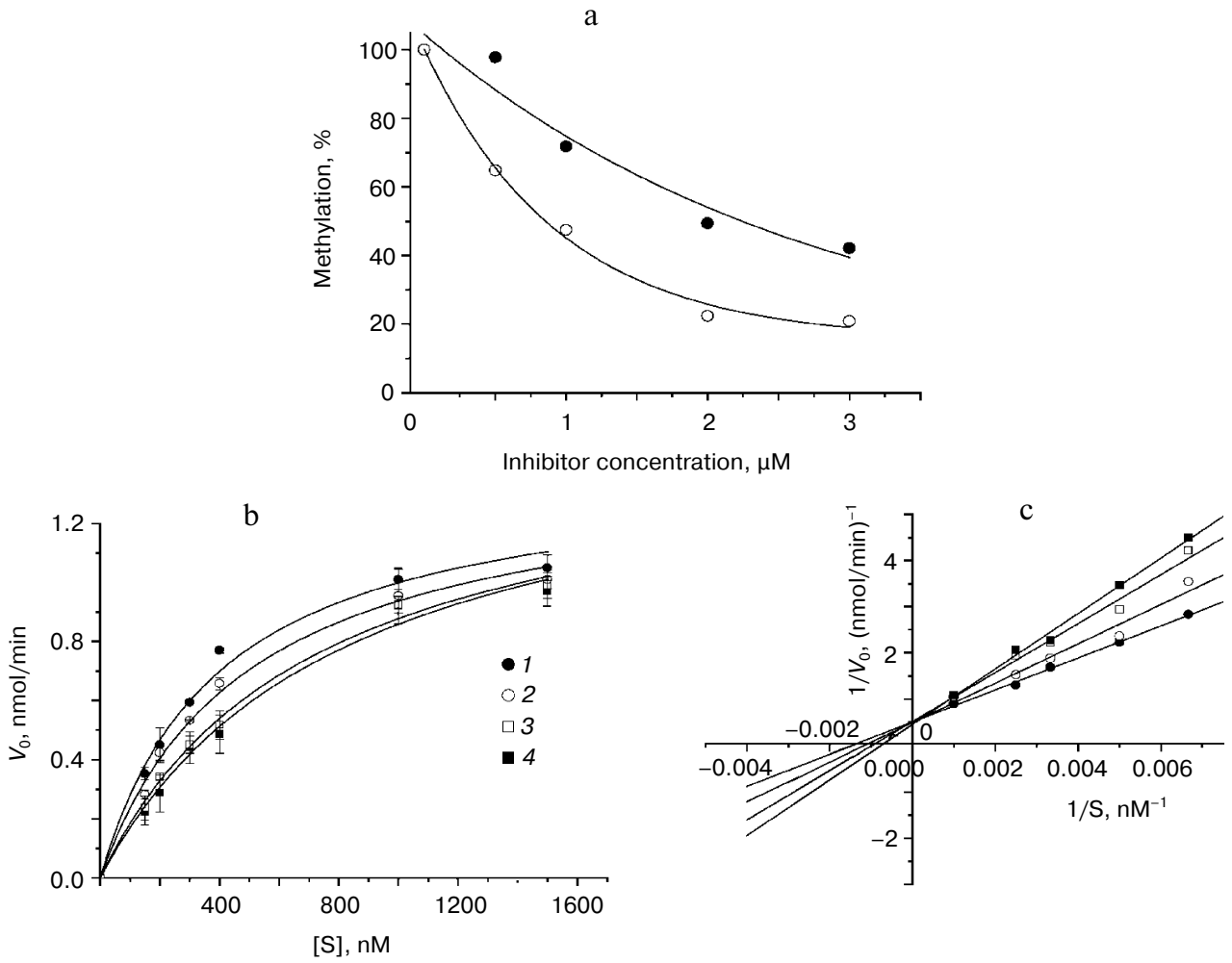


Fig. 5. Inhibition of Dnmt3a-CD/Dnmt3L complex by DNA containing **P** in target-cytosine position. a) Relation of the methylation rate (%) of DNA duplex 30**C**G/30G**C** (500 nM) with complex Dnmt3a-CD/Dnmt3L (50 nM) to concentration of inhibitors **P**G/G**M** (closed circles) and **MG**/G**P** (open circles). $C_{[CH_3\text{-}^3H]AdoMet}$, 1.3 μM. b) Dependence of initial rate of substrate 30**C**G/30G**C** methylation reaction on its concentration (150-1500 nM) at various concentrations of the inhibitor **MG**/G**P** (nM): 0 (*1*), 100 (*2*), 150 (*3*), and 200 (*4*). Concentration of Dnmt3a-CD/Dnmt3L was 150 nM; $C_{[CH_3\text{-}^3H]AdoMet}$, 2 μM. c) The same relations as in (b) linearized in Lineweaver–Burk double reciprocal coordinates. Designations are the same as in (b). Estimated value of inhibition constant K_i was equal to 240 ± 30 nM.

allow us to understand the molecular mechanism of zebularine action on C5-MTases *in vivo*.

Oligonucleotide inhibitors of C5-MTases containing **P** instead of target cytosine revealed similar features in the mechanism of action of Dnmt3a and prokaryotic C5-MTases: covalent intermediate forms during DNA methylation by means of a cysteine residue, and the contacts between C5-MTase and minor groove are required for its formation. But at the same time some particular characteristics of Dnmt3a were revealed: the low yield of conjugate, its instability to heating, and increased conjugate yield after activation of Dnmt3a by the regulatory factor Dnmt3L; these characteristics correlate with enzymatic properties of Dnmt3a and allow us to conclude that the active site of Dnmt3a differ from other C5-MTases active sites.

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