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SHORT COMMUNICATION

Dimeric bisbenzimidazoles inhibit the DNA methylation catalyzed by the murine Dnmt3a catalytic domain

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Abstract
When located in the DNA minor groove, dimeric bisbenzimidazoles DB(*n*) effectively inhibited *in vitro* the Dnmt3a catalytic domain (IC_{50} 5–77 μM). The lowest IC_{50} value was observed for compound DB(11) with an 11-unit methylene linker joining the bisbenzimidazole fragments. Increased time of incubation of DNA with DB(*n*) as well as the presence of AT-clusters in DNA enhances the inhibitory effect.

Keywords: DNA methyltransferase, inhibition, Hoechst 33258

Introduction

The DNA methyltransferase (MTase) Dnmt3a (EC 2.1.1.37) belongs to the family of mammalian Dnmt3[1,2]. MTase Dnmt3a can recognize a DNA CpG site and methylate the carbon 5 of cytosine residue (DNA-(cytosine C5)-methyltransferase (C5-MTase)) thus forming the *de novo* methylation profile[3]. Also, Dnmt3a together with MTase Dnmt1 is involved in maintenance DNA methylation[4–6]. X-ray studies revealed that complex *C*-terminal domain of Dnmt3a with its regulatory factor Dnmt3L exists as a 2:2 heterotetramer[7]. The importance of tetramer formation for S-adenosyl-L-methionine (AdoMet) binding, DNA binding and methylation has been shown[7]. The Dnmt3a-Dnmt3L complexes polymerize on the DNA forming nucleoprotein filament[8].

The correct distribution of methylated CpG sites in the genome is crucial for cell development. One of the mechanisms of tumour cell formation involves inactivation of tumour suppressor genes due to *de novo* hypermethylation of promoter CpG islands of these genes in the process of carcinogenesis[9–11]. As changes in the DNA methylation status are affected by MTase functioning, much attention was given to inhibition of these enzymes[12,13].

The DNA methylation process includes the formation of a covalent bond between a cysteine residue of the enzyme active site and the C6 position of the target cytosine in DNA[14]. At present the so called mechanism-based inhibitors (5-aza-2′-deoxycytidine, 5-fluo-2′-deoxycytidine, and 2-pyrimidinone-1-β-D-2′-deoxyribofuranoside) are the most effective inhibitors of C5-MTases[12,13]. In this case inhibition is irreversible and the formed covalent adducts either cannot be degraded or their degradation proceeds extremely slowly. However, because of incorporation of these compounds into DNA, they are very toxic and mutagenic *in vivo*. Therefore the search of new effective MTase non-nucleoside inhibitors is an actual task.

Several low molecular weight non-nucleoside MTase inhibitors are known to date: epigallocatechin 3-gallate and genistein (4′,5,7-trihydroxyisoflavone), whose IC_{50} towards Dnmt1 are 20 and 67 μM, respectively[15], disulfide derivatives of L-bromtyrosine (psammaplins; IC_{50} 3,4-18,8 nM for Dnmt1)[16], and L-tryptophan derivative RG108 [2-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-3-(1*H*-indol-3-yl) propionic acid] (IC_{50} 115 nM for DNA methyltransferase SssI (M.SssI))[17].

Based on the structure of the complex of prokaryotic MTase HhaI with DNA and S-adenosyl-L-homocysteine, most of the DNA-MTase contacts providing specific MTase binding to DNA occurs in the major groove of the double helix, and transfer of the methyl group to the target cytosine is accompanied by motion of the MTase

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catalytic loop towards the minor groove side of the DNA substrate[18]. Hence, DNA ligands capable of disturbing this motion may be effective MTase inhibitors. One of such ligands is Hoechst 33258, a bisbenzimidazole dye, which can bind to DNA AT sites in the minor groove with a binding constant 10^8–10^9 M^{-1} [19,20].

In this work we tested, as murine catalytic domain of Dnmt3a (Dnmt3a-CD) inhibitors, a series of dimeric benzimidazoles DB(*n*), namely, DB(1–5,7,11), differing in the length of methylene linkers joining bisbenzimidazole fragments and *bis*-HT(NMe), which contains two Hoechst 33258 fragments joined by means of a linker containing six methylene groups and one N(Me) group (Figure 1).

Materials and methods

General experimental

Reagents and solvents for syntheses were reagent-grade and were used without further purification. AdoMet, (32 mM stock solution) was from New England Biolabs. Buffer A (10 mM Tris-HCl (pH 7.9), 50 mM NaCl), B (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.0), 100 mM KCl, 1 mM ethylenediaminetetraacetic acid, and 0.2 mM 2-mercaptoethanol), C (10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 0.2 mM 2-mercaptoethanol), and Tango (*Fermentas, Vilnius, Lithuania*) were used.

Compound DB(5) was synthesized according to reported procedures[21]. Oligonucleotides 5′-FAM-CTGAATACTACTTGCGCTCTCTAACCTGAT, 5′ ATCAGGTTAGAGAGCGCAAGTAGTATTCAG, 5′-FAM-TG-GACACCACCTGCGCTCTCTGACCTGAC, and 5′-GTCAG-GTCAGAGAGCGCAGGTGGTGTCCAG were from Syntol (Moscow, Russia). The fluorescent label 6(5)-carboxyfluorescein (FAM) is joined with the oligodeoxyribonucleotides 5′-end with a $(CH_2)_6$-linker. DNA duplexes were prepared by mixing of FAM-labelled strand with 1.1-fold excess of complementary strand up to 5 μM duplex concentration in buffer A, heating to 85°C and allowing the sample to cool slowly over several hours to room temperature.

The murine *C*-terminal Dnmt3a-CD, which retained its catalytic activity in the absence of the *N*-terminal regulatory domain[22], was used. The plasmid pET28a containing the sequence encoding murine Dnmt3a-CD was provided by A. Jeltsch. Recombinant Dnmt3a-CD which contains amino acid residues 608-908 and the $(His)_6$ cluster at the *N*-terminus was expressed in the *Escherichia coli* BL21(DE3) strain (Novagen, Merck KGaA, Darmstadt, Germany). Dnmt3a-CD was purified to ~95% purity by affinity chromatography using Co^{2+} containing TALON affinity resin (Clontech, CA, USA) as described in[23]. Restriction endonuclease HhaI (R.HhaI) was purchased from Fermentas (Vilnius, Lithuania).

Hydrogen-nuclear magnetic resonance (^1H-NMR) Spectra; BrukerAMX-400 spectrometer (Rheinstetten, Germany), in (D_6)dimethyl sulfoxide; δ in ppm rel. to the residual solvent signal, J in Hz. Matrix assisted laser

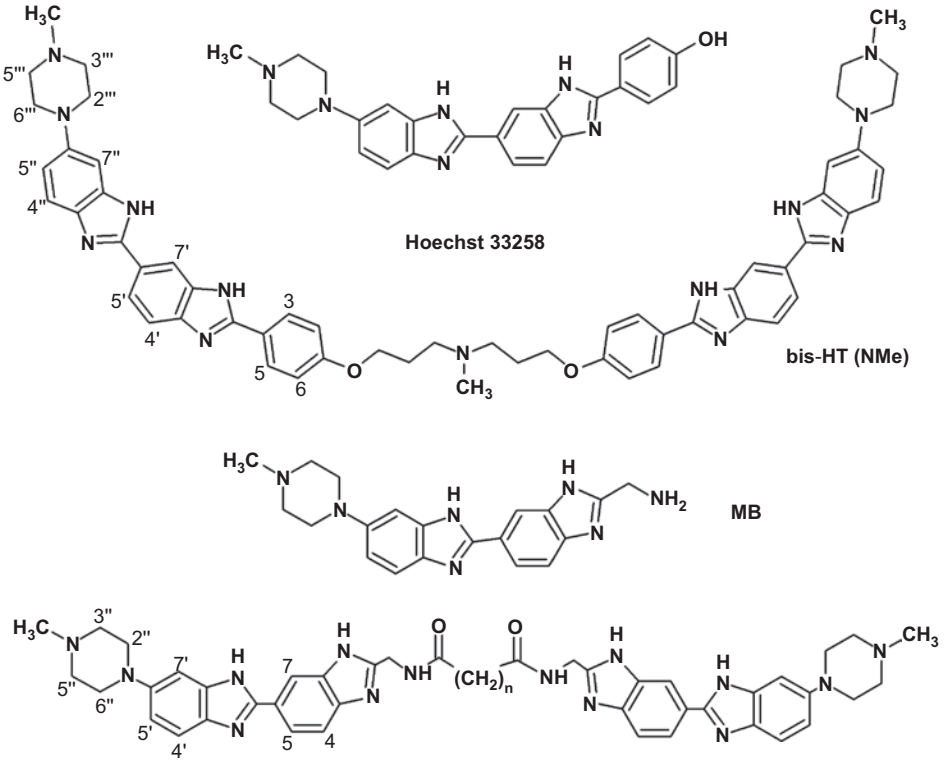


Figure 1. Chemical structures of dimeric bisbenzimidazoles DB(*n*), bis-Ht (NMe), monomeric bisbenzimidazoles (MB), and Hoechst 33258. The numbering of piperazine and benzimidazole hydrogen atoms is shown.

desorption ionisation time-of-flight mass spectrometry (MALDI TOF-MS): time-of-flow Vision-2000 mass spectrometer (ThermoBioanalysis, Hemel Hempstead, UK) with registration of positive ions; the matrix was 2,5-dihydroxybenzoicacid; N_2 laser, 337 nm; *m/z*.

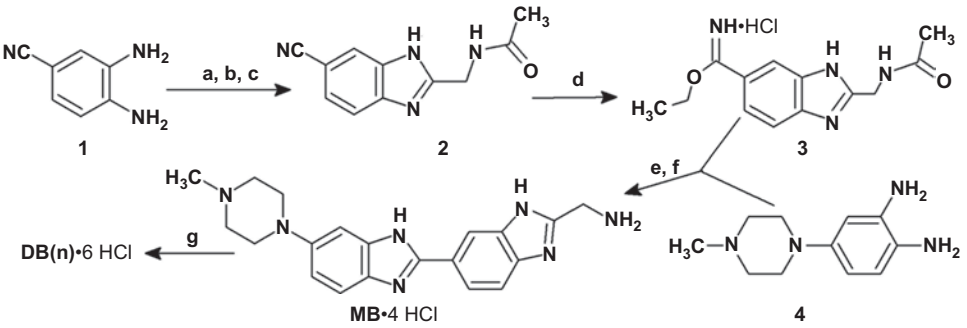
Synthesis of MB, DB(1–5, 7, 11), and bis-HT(NMe)

Compound DB(5) was synthesized as described in ref. 21. Monomeric bisbenzimidazole (MB) was prepared by coupling of 4-(4-methylpiperazin-1-yl)benzene-1-,2-diamine 4[24] and imidate 3 followed by removal of an *N*-acetyl protective group (scheme 1). Compound 3 was obtained by the Pinner reaction from nitrile 2, which was synthesized from 3,4-diaminobenzonitrile 1[25] by coupling/cyclization with *tert*-butyloxycarbonyl-glycine (Boc-Gly-OH). Dimerization of MB molecules by coupling with the corresponding reagents led to the formation of compounds DB(1–4,7,11; scheme 1). Compound *bis*-HT(NMe) was synthesized according to scheme 2 starting from the previously described diamine 8[21] and diimidate 7. Compound 7 was obtained by the Pinner reaction from dinitril 6. Compound 6 was prepared from 4-hydroxybenzonitrile 5 by coupling with 3-chloro-*N*-(3-chloropropyl)-*N*-methylpropan-1-amine. The structures of the synthesized compounds were confirmed by NMR spectroscopy and mass-spectrometry data.

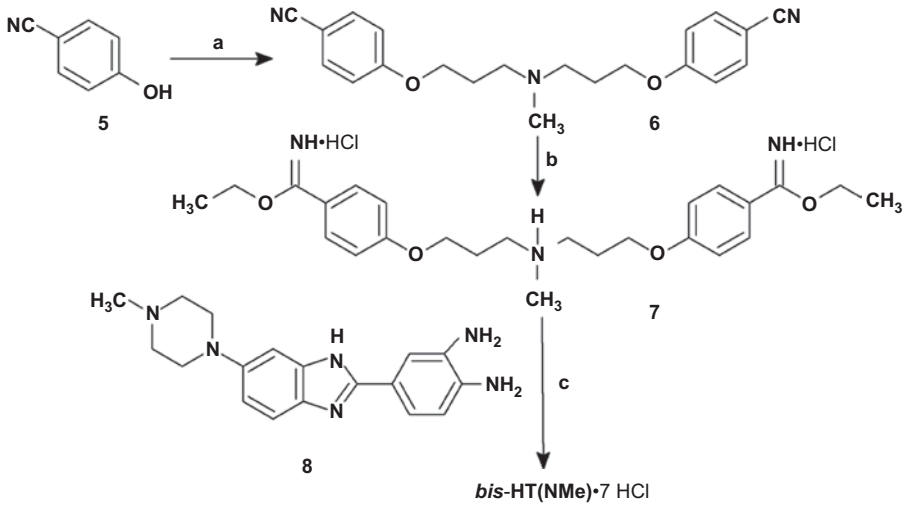
NMR and mass spectra characteristics of MB, DB(1–5, 7, 11), and bis-HT(NMe)

MB·4 HCl: ^1H-NMR (400 MHz, 97°C): δ 2.83 (3H, s, CH_3); 3.36 (4H, m, H(3‴, 5‴)); 3.58 (4H, m, H(2‴,6‴)); 4.40 (2H, s, CH_2NH_2); 7.19 (1H, d, *J* = 8.7, H5'); 7.24 (1H, s, H7'); 7.65 (1H, d, *J* = 9.3, H4'); 7.80 (1H, d, *J* = 8.1, H4); 8.23 (1H, d, *J* = 8.7, H5); 8.67 (1H, s, H7). MALDI TOF-MS (monoisotop.): calcd for $C_{20}H_{23}N_7$: 361.40; found: *m/z* $(M)^+$ 361.3.

DB(1)·6 HCl: ^1H-NMR (400 MHz, 32°C): δ 2.84 (6H, brs, CH_3); 3.23 (8H, m, H(3‴, 5‴)); 3.48 (2H, s, $COCH_2$); 3.54 (4H, m, H(2‴, 6‴)); 3.88 (4H, m, H(2‴, 6‴)); 4.79 (4H, d, *J* = 5.0, CH_2NH); 7.19 (2H, brs, H7'); 7.34 (2H, d, *J* = 9.3, H5'); 7.69 (2H, d, *J* = 8.7, H4'); 7.92 (2H, d, *J* = 8.7, H4); 8.34



Scheme 1. Synthesis of DB(1–5,7,11). Reagents and conditions: (a) iBuOCOCl, NMM, Boc-Gly-OH; (b) AcOH, 65–70°C, 1 h; (c) AcOH, 120°C, 4 h, 58% (for a–c); (d) HCl_{gas}/EtOH, 0–4°C, 1 h, 23°C, 3 days; (e) AcOH/EtOH, 95°C, 1 h, N_2, 78% (for d–e); (f) HCl_{conc}, 105°C, 20 min, 89%; (g) for DB(1,2): DMF, DIPEA, BOP, HOOC-(CH_2)n-COOH (*n* = 1,2), 0°C, 1 h, 23°C, 1 day, HCl/MeOH, DB(1), 22%, DB(2), 24%; for DB(3–5,7,11): DMF, Et_3N, XOOC-(CH_2)n-COOX (*X* = Np; Pfp; Su; *n* = 3,4,5,7,11), HCl/MeOH, DB(3), 73%, DB(4), 68%, DB(5), 51%, DB(7), 78%, DB(11), 61%. All DB(*n*) were purified by refluxing in methanol followed by cooling and filtration of the target precipitate. AcOH, acetic acid; Boc-Gly-OH, *tert*-butyloxycarbonyl-glycine; BOP, (benzotriazole-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; DIPEA, *N*,*N*-diisopropylethylamine; *DMF*, dimethylformamide; Et3N, triethylamine; EtOH, ethanol; iBuOCOCl, isobutyl chloroformate; MeOH, methanol; NMM, *N*-methylmorpholine; Np, p-nitrophenyl; Pfp, 2,3,4,5,6-pentafluorophenÑƒl; Su, N-oxysuccinimide.



Scheme 2. Synthesis of bis-HT(NMe). Reagents and conditions: (a) $Cl(CH_2)_3N(CH_3)(CH_2)_3Cl$, NaH, DMF; (b) HCl, EtOH; (c) AcOH, N_2, 120°C, HCl/MeOH, 20%. bis-HT(NMe) was purified by recrystallization from a mixture of MeOH-H_2O-HCl.

(2H, d, J=8.7, H5); 8.75 (2H, s, H7); 9.19 (2H, *t*, J=5.3, CONH); 11.23 (2H, brs, NH(Bim)). MALDI TOF-MS (monoisotop.): calcd for $C_{43}H_{16}N_{14}O_2$: 790.92; found: *m/z* $(M + H)^+$ 793.4, $(M + Na + K)$ + 855.7.

DB(2)·6 HCl: ^1H-NMR (400 MHz, 32°C): δ 2.63 (4H, s, CH_2CO); 2.84 (6H, brs, CH_3); 3.23 (8H, m, H(3″, 5″)); 3.54 (4H, m, H(2″, 6″)); 3.87 (4H, м, (2″, 6″)); 4.74 (4H, d, J=5.6, CH_2NH); 7.17 (2H, brs, H7′); 7.32 (2H, d, J=8.7, H5′); 7.68 (2H, d, J=9.3, H4′); 7.87 (2H, d, J=8.7, H4); 8.32 (2H, d, J=8.7, H5); 8.72 (2H, s, H7); 8.99 (2H, *t*, J=5.6, CONH); 11.24 (1H, brs, NH(Bim)). MALDI TOF-MS (monoisotop.): calcd for $C_{44}H_{48}N_{14}O_2$: 804.94; found: *m/z* $(M + H)^+$ 806.3.

DB(3)·6 HCl: ^1H-NMR (400 MHz, 23°C): δ 1.86 (2H, quintet, J=7.5, $CH_2CH_2CH_2$), 2.33 (4H, *t*, J=7.5, CH_2CO), 2.84 (6H, d, J=3.7, CH_3), [3.38 (8H, m, H(3″,5″)), 3.58 (8H, m, H(2″,6″)—at 97°C)], 4.72 (4H, d, J=5.0, CH_2NH), 7.18 (2H, brs, H7′), 7.34 (2H, dd, J=1.9, J=9.3, H5′), 7.70 (2H, d, J=9.3, H4′), 7.94 (2H, d, J=8.7, H4), 8.34 (2H, d, J=8.7, H5), 8.73 (2H, s, H7), 8.97 (2H, *t*, J=5.3, CONH), 11.13 (2H, brs, NH(Bim)). MALDI TOF-MS (monoisotop.): calcd for $C_{45}H_{50}N_{14}O_2$: 818.88; found: *m/z* $(M + H)^+$ 820.2.

DB(4) 6 HCl: ^1H-NMR (400 MHz, 32°C): δ 1.60 (4H, м, CH_2CH_2CO), 2.32 (4H, м, CH_2CO), 2.83 (6H, brs, CH_3), 3.24 (8H, m, H(3″,5″)), 3.53 (4H, m, H(2″,6″)), 3.86 (4H, m, H(2″,6″)); 4.77 (4H, d, J=5.0, CH_2NH), 7.18 (2H, brs, H7′), 7.33 (2H, d, J=9.3, H5′), 7.69 (2H, d, J=8.7, H4′), 7.96 (2H, d, J=8.7, H4), 8.47 (2H, d, J=9.3, H5); 8.84 (2H, s, H7), 8.95 (2H, *t*, J=5.3, CONH), 11.46 (1H, brs, NH(Bim)). MALDI TOF-MS (monoisotop.): calcd for $C_{46}H_{52}N_{14}O_2$: 832.91; found: *m/z* $(M + H)^+$ 833.7.

DB(5) 6 HCl: ^1H-NMR (400 MHz, 32°C): δ 1.32 (2H, quintet, J=7.5, $CO(CH_2)_2CH_2$), 1.58 (4H, quintet, J=7.5, $COCH_2CH_2$), 2.26 (4H, *t*, J=7.5, $COCH_2$,), 2.85 (6H, s, CH_3), [3.39 (8H, m, H(3″,5″)); 3.78 (8H, m, H(2″,6″)—at 84°C], 4.63 (4H, d, J=5.6, CH_2NH,), 7.20 (2H, d, J=1.9, H7′), 7.31 (2H, dd, J=1.9, J=8.7, H5′), 7.68 (2H, d, J=8.7, H4′), 7.89 (2H, d, J=8.7, H4), 8.26 (2H, d, J=8.7, H5), 8.66 (2H, s, H7), 8.68 (2H, *t*, J=5.3, CONH), 11.03 (1H, brs, NH(Bim)). MALDI TOF-MS (monoisotop.): calcd for $C_{47}H_{54}N_{14}O_2$: 847.02; found: *m/z* $(M + H)^+$ 848.3.

DB(7) 6 HCl: ^1H-NMR (400 MHz, 32°C): δ 1.28 (6H, m, $CO(CH_2)_2(CH_2)_3$), 1.55 (4H, m, $COCH_2CH_2$), 2.23 (4H, *t*, J=7.5, $COCH_2$), 2.83 (6H, s, CH_3), [3.38 (8H, m, H(3″,5″)); 3.55 (8H, m, H(2″,6″))—at 84°C], 4.58 (4H, d, J=5.0, CH_2NH), 7.19 (2H, s, H7′), 7.26 (2H, d, J=8.7, H5′), 7.66 (2H, d, J=8.7, H4′), 7.80 (2H, d, J=8.1, H4), 8.20 (2H, d, J=8.1, H5), 8.60 (4H, m, CONH, H7), 11.19 (1H, brs, NH(Bim)). MALDI TOF-MS (monoisotop.): calcd for $C_{49}H_{58}N_{14}O_2$: 875.08; found: *m/z* $(M + H)^+$ 876.5.

DB(11)·6 HCl: ^1H-NMR (400 MHz, 23°C): δ 1.22 (14H, m, $CO(CH_2)_2(CH_2)_7$), 1.52 (4H, m, $COCH_2CH_2$), 2.23 (4H, *t*, J=7.2, $COCH_2$), 2.83 (6H, brs, CH_3), 3.21 (8H, m, H(3″, 5″)), 3.53 (4H, m, H(2″,6″)), 3.88 (4H, m, H(2″,6″)), 4.66 (4H, d, J=5.0, CH_2NH), 7.19 (2H, s, H7′), 7.33 (2H, dd, J=1.2, J=8.7, H5′), 7.70 (2H, d, J=8.7, H4′), 7.91 (2H, d, J=8.7, H4), 8.34 (2H, d, J=8.7, H5), 8.73 (2H, s, H7), 8.78 (2H, *t*, J=5.3, CONH), 11.19 (2H, brs, NH(Bim)). MALDI TOF-MS (monoisotop.): calcd for $C_{53}H_{66}N_{14}O_2$: 931.18; found: *m/z* $(M + H)^+$ 931.8.

bis-HT(NMe)·7 HCl: ^1H-NMR (400 MHz, 23°C): δ 2.25 (4H, quint, J=6.5, OCH_2CH_2); 2.85 (9H, brs, CH_3); 3.12 (8H, m, H(3‴, 5‴)); 3.35 (4H, m, CH_2N); 3.36 (8H, m, H(2‴, 6‴)); 4.23 (4H, m, OCH_2); 7.18 (6H, m, H(2, 6′, 7″)); 7.28 (2H, dd, J=1.9, J 9.3, H5″); 7.67 (2H, d, J=9.3, H4″); 7.86 (2H, d, J=8.7, H4′); 8.24 (2H, d, J=8.7, H5′); 8.30 (4H, d, J=8.7, H(3, 5)); 8.62 (2H, s, H7′). MALDI TOF-MS (monoisotop.): calcd for $C_{57}H_{61}N_{13}O_2$: 960.09; found: *m/z* $(M + H)^+$ 961.0.

Enzyme inhibition assay

Duplexes A or B (300 nM) were incubated in the presence of increasing concentrations of a DB(*n*) inhibitor (0.1–200 μM) in buffer B (for Dnmt3a-CD) or C (for M.SssI) for 3 days at 4°C or for 10 min at 25°C. Dnmt3a-CD (2 μM of monomers) or M.SssI (2 μM) and AdoMet (25 μM) were added to the reaction mixtures and the mixtures were incubated for 40 min at 37°C. Reaction mixtures lacking either the enzyme or the inhibitor were used as controls. The DNA was precipitated with ethanol (EtOH) in the presence of 0.4 M sodium acetate. The precipitate was washed with 80% EtOH and evaporated on a SpeedVac to remove remained EtOH. The methylation was studied by protection of the methylated DNA from cleavage by 2U of R.HhaI in the 20 μL Tango buffer for an hour at 37°C. The cleavage products were suspended in 10 μL 80% formamide and separated in 20% polyacrylamide gel under denatured conditions (7 M urea) followed by gel imaging on a FUJIFILM FLA-3000 device.

The methylation % (M) was determined as the ratio of methylated to total DNA based on the intensities of the corresponding bands in gel using the Image Quant 5.0 program. To determine inhibitor concentration which reduced enzyme activity by 50% (IC_{50}) the dependence of relative methylation percentage (*R*, %) versus inhibitor concentration were plotted. *R* values were calculated from the formula:

$$\frac{\left(M - M^{\text{enzyme free}}\right)}{\left(M^{\text{inhibitor free}} - M^{\text{enzyme free}}\right)} \times 100$$

The curves were subjected to regression analysis in Origin 6.0 program using simple exponential function:

$$R = R_0 + A \cdot e^{[1]/t},$$

where input parameters were *R* and inhibitor concentration, [I] (μM); R_0, A, and *t* were dependent parameters for fitting. All data were fitted with correlation coefficients more than 0.95. The IC_{50} values were then determined by interpolation. For DB(4,5) when IC_{50} values exceed the inhibitor concentration range, they were estimated by extrapolation of the above mentioned function to 50% inhibition. The values of standard errors were calculated using 3–6 independent experiments.

Results and discussion

The inhibitory activities of DB(*n*) and *bis*-HT(NMe) were studied in the reaction of Dnmt3a-CD catalyzed methylation of a 30-mer DNA duplex: 5′-FAM-

CTGAATACTACTTG**C̲G**CTCTCTAACCTGAT, 3′-GACTTATGATGAAC**GC̲**GAGAGATTGGACTA (A) containing a CpG site (shown in bold; cytosine residues to be methylated are underlined, clusters of AT pairs are indicated by larger letters) and a fluorescent FAM label at the 5′-end of the upper strand. The pre-incubation time of DB(*n*) with the 30-mer DNA duplex A, varied from 10 min to 3 days. Compounds *bis*-HT(NMe) and DB(1–5,7,11) demonstrated inhibitory activity at micromolar concentrations (Table 1). The increase of incubation time resulted in 3–9-times decrease of IC_{50} value.

Compound *bis*-HT(NMe) could form with AT-rich DNA complexes of three types depending mainly on the incubation time[26]. Type I complexes were formed by *bis*-HT(NMe) in the open linear form; type II complexes were formed by intramolecular sandwiches and type III, by dimers of intramolecular sandwiches. Within complexes I, II, and III, the *bis*-HT(NMe) occupies on DNA 10–11, 5–6, and 2–3 bp, respectively[26]. In the case of immediate addition to *bis*-HT(NMe) of a large DNA excess the formation of type III complex was observed[26]. This type of complex is metastable and it rearranges slowly to the mixture of type I and II complexes. At *bis*-HT(NMe) concentrations used in the experiments type I and II complexes were prevalent in the solution in 3 days, after thermodynamic equilibrium had been set. The inhibitory effect of *bis*-HT(NMe) (Table 1) was higher in the case of types I and II complexes (3 days incubation) than it was in the case of type III complex (10 min incubation). One can suggest that compounds DB(1–5,7,11) could form types I and II complexes which displayed higher inhibitory effects under the conditions of long-term incubation.

It is noteworthy that MB (Figure 1) only negligibly inhibited Dnmt3a-CD even under prolonged incubation with DNA (the enzymatic activity was reduced by 7% in the presence of 200 μM MB; data not shown). This fact supported the hypothesis that it was dimerization of MB molecules that provided the formation of DB(*n*) molecules with high inhibitory activity.

The observed inhibitory activities of DB(*n*) were affected by the length of methylene linkers (Table 1). DB(*n*) with the shortest (DB(1–3)) and the longest (DB(11)) methylene linkers showed the lowest IC_{50} values.

It is known that a bisbenzimidazole molecule binds preferably to the DNA fragment containing two AT or TA pairs[19]. For binding of DB(*n*) two such fragments are required. Several AT-clusters in duplex A located at different distances relative to each other can be involved in the DB(*n*) binding. One can suppose that the most effective binding occurs when the distance between AT-clusters corresponds to the length of the methylene linkers joining bisbenzimidazole fragments. For example, the 5′-TACTTG**C̲G**/3′-ATGAAC**GC̲**…fragment of duplex A would be preferable for binding to DB(1–3) and the 5′-TACTACTTG**C̲G**/3′-ATGATGAAC**GC̲** fragment of duplex A would be preferable for binding to DB(11). It is likely that a high IC_{50} value for binding of DB(4,5) may be explained by a non-optimal distance between AT-clusters in duplex A (Table 1). Within the tested DB(*n*) bisbenzimidazoles, DB(11) was the most

Table 1. Inhibition of the methylation of duplex A by Dnmt3a-CD in the presence of DB(*n*).

	IC_{50}, (μM) ± SEM[a]	
	Incubation time of the DNA-inhibitor mixture	
Compound	10 min	3 days
DB(1)	33.9 ± 9.8	12.5 ± 5.5
DB(2)	35.5 ± 9.2	9.8 ± 1.6
DB(3)	150 ± 40	11.2 ± 3.0
DB(4)	~300[b]	38.0 ± 7.0
DB(5)	~700[b]	77.8 ± 12.0
DB(7)	53.0 ± 9.5	18.7 ± 4.8
DB(11)	18.0 ± 5.6	5.0 ± 1.5
		4.6 ± 0.6[c]
		49.0 ± 2.0[d]
bis-HT(NMe)	13.8 ± 3.1	4.6 ± 1.8

[a]Standard mean error of 3–6 assays.
[b]Were estimated by extrapolation of experimental data to 50% inhibition.
[c]Methylation by DNA methyltransferase SssI.
[d]Methylation of duplex B.

effective inhibitor with the IC_{50} value close to that of *bis*-HT(NMe) (Table 1). The distance between bisbenzimidazole fragments in these molecules is also similar.

Compound *bis*-HT(NMe) occupies 10–11 bp in the DNA minor groove in open linear form[26]. Therefore, high inhibitory properties of DB(11) and bis-HT(NMe) seem to be due to the open linear conformation of DB(*n*) in the complex with DNA.

For evaluation if the inhibition effect depends on the presence of AT-clusters in DNA, we obtained duplex B lacking AT-clusters: 5′-FAM-TGGACACCACCT-G**C̲G**CTCTCTGACCTGAC, 3′-GACCTGTGGTGGAC**GC̲**-GAGAGACTGGACTG (B). The IC_{50} value of the DB(11) complex with duplex B (49.0 ± 2.0 μM) was one order of magnitude higher than that of the complex of DB(11) with duplex A (5.0 ± 1.5 μM). Therefore, AT-clusters within DNA are necessary for the effective inhibition of the DNA methylation by Dnmt3a. However, weak inhibition might be possible for any nucleotide sequence as it is observed for duplex B whose IC_{50} value is still in micromolar range.

With the goal to explain the mechanism of Dnmt3a inhibition we took into account the minor groove localization of DB(*n*). This assumption is based on the fact that Hoechst 33258, a MB derivative, is known to be located in the minor groove of the $d(CGCGAATTCGCG)_2$ double helix[19,27]. In addition, disposition of Hoechst 33258 in the DNA minor groove was demonstrated for type I and II complexes with poly(dG-dC)·poly(dG-dC)[28]. Recently we found that the presence of a bulky benzo[*a*]pyrene residue in the DNA minor groove caused a dramatic decrease in prokaryotic MTases SssI and HhaI methylating capacity because of distortion of DNA contacts with the enzyme catalytic loop[29,30]. Superimposition of the DNA methyltransferase HhaI (M.HhaI–DNA) complex structure onto the Dnmt3a-CD structure showed high similarity of M.HhaI and Dnmt3a-CD catalytic loop conformations[7]. Thus, the observed DB(*n*) inhibitory effects might be explained by perturbation of minor groove-Dnmt3a loop interactions due

to the presence of a bulky ligand. This type of inhibition may be considered as the substrate protection mechanism.

In the case of addition of DB(11) inhibitor to the pre-incubated DNA-Dnmt3a-CD complex we did not observe significant reduce of methylation activity (data not shown). This fact suggests competition between DB(*n*) molecules and enzyme for DNA binding to DNA minor groove.

Also, we studied if the observed inhibitory effect of DB(*n*) was only inherent for Dnmt3a-CD. The interaction of M.SssI with duplex A was studied in the presence of DB(11) (Table 1). We showed that DB(11) inhibited M.SssI (IC_{50} 4.6 ± 0.6 μM) at the same concentrations as Dnmt3a-CD (IC_{50} 5.0 ± 1.5 μM). It is noteworthy that MBs could inhibit human topoisomerase I[31], DNA repair enzyme O6-alkylguanine-DNA alkyltransferase[32], and (A)BC excinuclease from *E. coli*[33] and DB(5) could inhibit HIV integrase[21]. We suppose that inhibition of the above DNA operating enzymes is attained via the substrate protection mechanism.

To summarize, we first found that compounds DB (1–5,7,11) are *in vitro* inhibitors of Dnmt3a-CD at low micromolar concentrations which is consistent with concentrations reported by others for different inhibitors of C5-MTases[15,34]. It is notable that the same range of concentrations (5–50 μM) of non-nucleoside MTase inhibitor was used in the case of cell culture assays[15]. It is noteworthy that the use of DB(*n*) as *in vivo* MTase inhibitors is promising due to their capacity to penetrate through cell and nuclear membranes of living cells and bind to chromatin[35].

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Declaration of interest

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