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Phosphorothioation of Oligonucleotides Strongly Influences the Inhibition of Bacterial (M.HhaI) and Human (Dnmt1) DNA Methyltransferases

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The cytidine analogue 5-fluoro-2′-deoxycytidine (dC^F) is a mechanism-based inhibitor of DNA methyltransferases. We report the synthesis of short 18-mer dsDNA oligomers containing a triple-hemimethylated CpG motive as a recognition sequence for the human methyltransferase Dnmt1. The DNA strands carry within these CpG islands dC^F building blocks that function as mechanism-based inhibitors of the analyzed methyltransferases. In addition, we replaced the phosphodiester backbones at defined positions by phosphorothioates. These hypermodified DNA strands were investigated as inhibitors of the DNA methyltransferases M.HhaI and Dnmt1 in vitro. We could show that both methylases behave substantially differently in respect to the amount of DNA backbone modification.

Introduction

The enzymes M.HhaI and Dnmt1 are DNA-cytosine methyltransferases that catalyze the transfer of the activated methyl group of *S*-adenosylmethionine (SAM) to the C5 position of cytosine.[1,2] M.HhaI is the methyltransferase originally isolated from *Haemophilus haemolyticus* in which it serves as part of a restriction–modification system that defends the bacteria against infection by bacteriophages. It methylates the internal cytosine in the recognition sequence 5′-GCGC-3′ to give 5′-GC^MGC-3′.[3–6] The human Dnmt1 is termed maintenance methyltransferase because it preferentially methylates hemimethylated DNA in 5′-CG-3′ islands after DNA replication.[7–11] These CpG-islands are epigenetic markers involved in various biological and developmental processes such as genomic imprinting,[12,13] X-chromosome inactivation,[14,15] gene regulation,[16–18] genetic diseases[19,20] and carcinogenesis.[21–23] Cancers, for example, frequently feature abnormally high levels of methylation of their CpG-rich promoter regions. Often this cancer-associated hypermethylation downregulates tumor suppressor genes and thus contributes to tumor formation and progression.[24,25] Therefore DNA-demethylating agents especially inhibitors of Dnmt1 might be able to interfere with the methylation process, which might lead to the reactivation of suppressed genes.[26–30] Among the best-studied Dnmt1 inhibitors are 5-azadC,[31] 5-azaC,[32] zebularine,[33] and dC^F.[34] After addition to cells they are probably converted to their corresponding triphosphates and incorporated into the cellular genome, where they act as suicide substrates for Dnmt1. Ribose-based inhibitors first have to be transformed to their deoxyribose form and are therefore also incorporated into RNA, which reduces their efficacy. In DNA, these bases are able to form a covalent bond with the catalytically essential SH group of a Cys amino acid in the active site of Dnmt1.[35–38] Because of the formation of long-lived, irreversible DNA–protein adducts, all these nucleoside inhibitors are cytotoxic.[39–41] To overcome this disadvantage, Szyf and co-workers synthesized backbone-modified hairpin oligodeoxynucleotides (ODNs) as Dnmt1 antagonists.[42,43] We published recently the synthesis and properties of oligodeoxynucleotide-dumbbell structures in which we incorporated the inhibitor 5-azadC. These dumbbells efficiently inhibited Dnmt1 in vitro. After treatment of C2C12 myoblast cells with the dumbbells, they showed colocalization with Dnmt1.[44]

Here we report the ability of short highly modified double-stranded oligonucleotides to inhibit the prokaryotic methyltransferase M.HhaI and the eukaryotic methyltransferase Dnmt1 in vitro. The concept is depicted schematically in Figure 1. We show that additional phosphorothioate backbone modifications either reduce or strongly increase the potency of the suicide substrates.

The ODNs prepared for this study contain a hemimethylated enzyme-recognition sequence that was already shown to be a target for these enzymes.[44] Opposite the dC^MdG islands dC^F nucleobases were placed as suicide substrate in the counterstrand, as is shown in Figure 1. The full sequences are listed in Figure 2.

In order to study how a phosphorothioate backbone, which is needed to increase the lifetime of the oligonucleotide inhibitors in serum and cells would influence their interaction with the methyltransferase, we prepared a series of phosphorothioate-modified DNA double strands (shown in green in Figure 2). The double strands contain either no phosphorothioates, half phosphorothioates, or all of the phosphodiesters were replaced by phosphorothioates.

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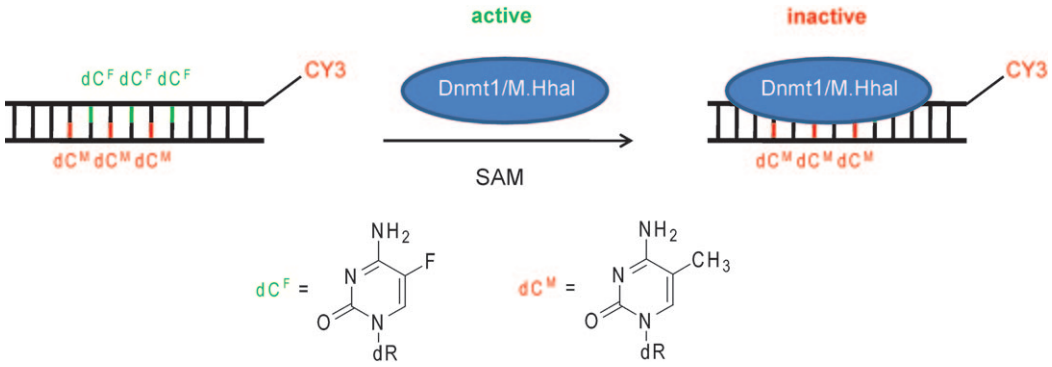

Figure 1. Inhibition of Dnmt1 and M.HhaI by short double-stranded oligonucleotides. dC^F = 5-fluoro-2′-deoxycytidine (green), dC^M = 5-methyl-2′-deoxycytidine (red), CY3 = cyanine-dye label, dR = 2′-deoxyribose.

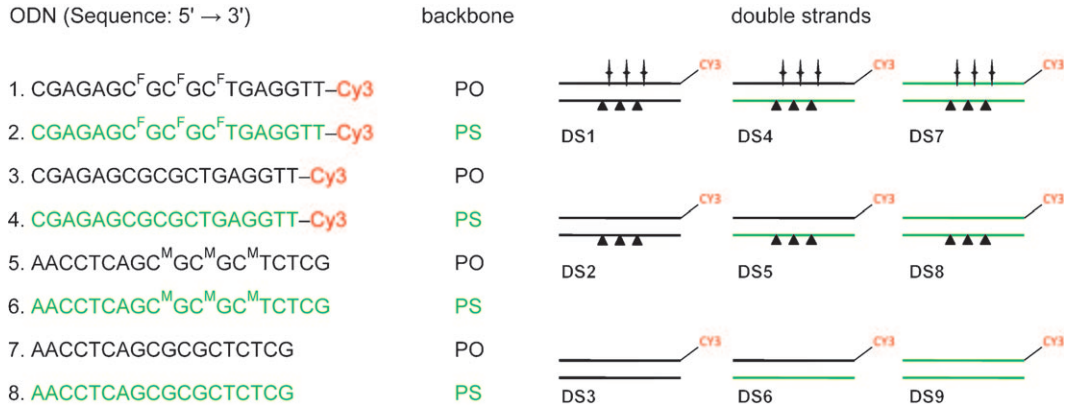

Figure 2. Sequence of the single strands (left). C^F/ϟ = dC^F; C^M/▲ = dC^M; PO (black) = phosphodiester backbone; PS (green) = phosphorothioate backbone. ODNs were mixed in equimolar amounts and hybridized to give the double strands (right).

Results

Electrophoretic mobility shift assays with M.HhaI

M.HhaI was incubated with equal amounts of **DS1**–**9** together with the cofactor SAM at 37 °C for 2 h. The reaction was subsequently analyzed by denaturing PAGE as shown in Figure 3 (for details see the Experimental Section).

To show that all the crosslinks present in the gels are formed through reaction with an active enzyme, M.HhaI was in a control experiment inactivated by heating the solution for 10 min at 90 °C (lane 5 in all three gels). From the gel experiments depicted in Figure 3 one can conclude that DNA–enzyme crosslinks were only formed between **DS1**, **4**, and **7** with the M.HhaI enzymes (lane 1). These are the duplexes containing the dC^F inhibitors. They all bind stably to the methyltransferase irrespective of the presence of the phosphorothioate status. The formation of double bands in the gels can be explained by a partial dissociation of the DNA duplex, which is bound only via one strand to the enzyme. The fact that M.HhaI forms a stable complex with DNA that contains dC^F even in the absence of the SAM cofactor is in agreement with literature data (lane 2).[45] For **DS2**, **3**, **5**, **6**, **8**, and **9** no additional shifted bands were detected; this shows that they do not bind and react with the protein (lanes 3, 4 and 6). To compare the amount of complex formed, the reactions of **DS1**, **4**, and **7** with the enzyme M.HhaI were analyzed directly on the same gel (Figure 4). The three inhibitors that contain DNA strands differ only in the amount of backbone modification. Whereas **DS1** has a complete natural phosphodiester backbone, **DS4** features a methylated strand that is fully phosphorothioate modified. In **DS7** all phosphodiesters in the whole duplex were replaced by phosphorothioates. The data presented in Figure 4 show that, in the

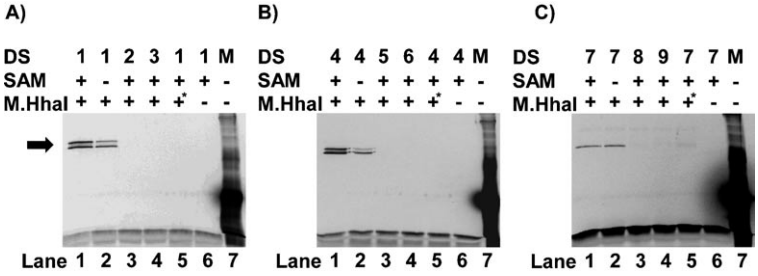

Figure 3. EMSA : Fluorescence detection of DNA–enzyme complexes. 13.4 pmol of M.HhaI were incubated either with 3.2 pmol of A) **DS1**–**3**, B) **DS4**–**6** or C) **DS7**–**9**. The arrow indicates DNA covalently bound to the enzyme. M = molecular weight marker, * indicates that M.HhaI was inactivated by heat before incubation. Crosslinks could also be stained with Coomassie (data not shown).

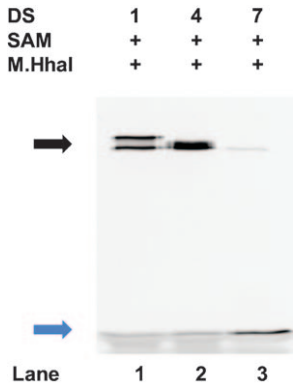

Figure 4. EMSA: Comparison of complex formation of **DS1**, **4**, and **7** with M.HhaI by fluorescence detection. DNA (3.2 pmol) was incubated with M.HhaI (50 U). The black arrow indicates DNA that is covalently bound to the enzyme, the blue one indicates free DNA.

case of a fully phosphorothioate backbone, the band stemming from the enzyme–DNA crosslink has nearly vanished; this proves that normal phosphodiesters are needed for efficient crosslink formation. The phosphorothioate-only duplex **DS7** reacts inefficiently with the protein.

Restriction protection assay with M.HhaI

In order to investigate the interaction of the DNA double-stranded inhibitors in more detail, we studied the direct inhibition of the DNA methylation reaction. To this end we performed the assay that is depicted in Figure 5. We used λ DNA as the substrate for the enzyme. After active methylation, λ DNA is not a substrate for the restriction enzyme HhaI anymore. For the assay we preincubated methyltransferase M.HhaI with cofactor SAM and the respective ODNs for 75 min at 37 °C. Then λ DNA was added and the methylation reaction mixture was incubated for an additional 4 h. The reaction mixture was treated with the restriction enzyme HhaI and subsequently analyzed by native PAGE (Figure 6). In case of methylation inhibition, λ DNA was not methylated and the restriction enzyme (HhaI) was able to cut λ DNA. In the presence of active M.HhaI, however, λ DNA was methylated at all dC sites, which in turn stops the restriction enzyme HhaI.

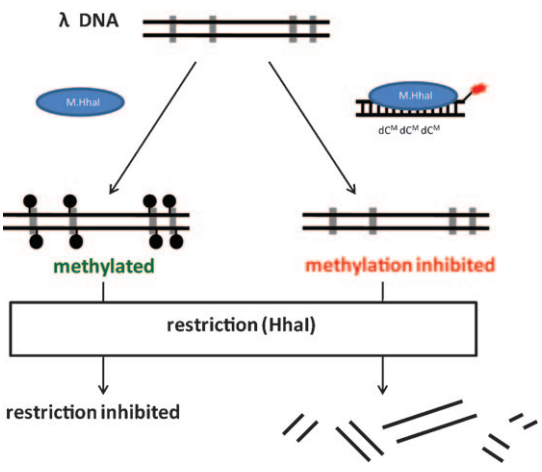

Figure 5. Restriction protection assay for the bacterial methyltransferase M.HhaI.

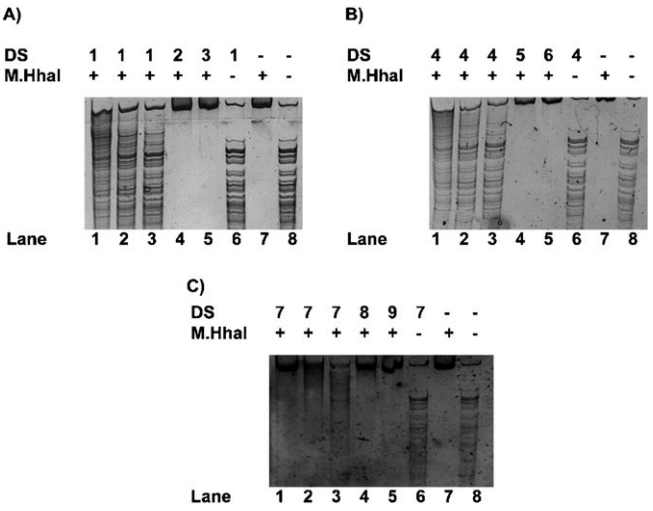

Figure 6. Fluorescence detection: A) 4.0, 8.0 and 16.0 pmol of **DS1** resp. 16.0 pmol **DS2** and **DS3**, B) 4.0, 8.0, and 16.0 pmol of **DS4** resp. 16.0 pmol **DS5** and **DS6**, and C) 4.0, 8.0, and 16.0 pmol of **DS7** resp. 16.0 pmol **DS8** and **DS9** were treated with 6.7 pmol of M.HhaI. DNA ladders implicate a positive restriction reaction and therefore inhibition of M.HhaI.

We added the inhibitor strands **DS1**, **4**, and **7** to the assay mixture in three different concentrations, substoichiometric (lane 1), stoichiometric (lane 2), and in a concentration double that of the methyltransferase (lane 3). As a control experiment, we performed the restriction assay also in the presence of **DS2** and **DS3**, which were shown to be unable to bind to the methyltransferase. Indeed, these control strands showed no inhibitory effects in the restriction assay (lanes 4 and 5). In contrast and in agreement with the EMSA experiments, **DS1** and **DS4** fully inhibit M.HhaI when applied stoichiometrically. If the inhibitor double strands are used substoichiometrically, residual methyltransferase activity could be detected, which is expected for the suicide inhibitors. In agreement with the gel-shift data, the fully phosphorothioate-modified duplex **DS7** is unable to fully inhibit M.HhaI even at high concentrations; this proves that full replacement of the phosphodiester backbone by phosphorothioates strongly reduces the binding of the duplex to the enzyme.

Electrophoretic mobility shift assays with Dnmt1

In order to investigate the human methyltransferase Dnmt1, we explored how this enzyme might be inhibited by our DNA double strands. To this end, Dnmt1 (7.5 pmol) was incubated with equal amounts (4.0 pmol) of each oligonucleotide for 2 h at 37 °C after addition of the cofactor SAM. The reaction was subsequently analyzed by SDS PAGE (Figure 7). The control experiments were the same as those performed with M.HhaI (lanes 5, 6 and 7). Interestingly, with the concentrations of DNA and enzyme used here (see the Experimental Section for details), no crosslink between the DNA and Dnmt1 could be de-

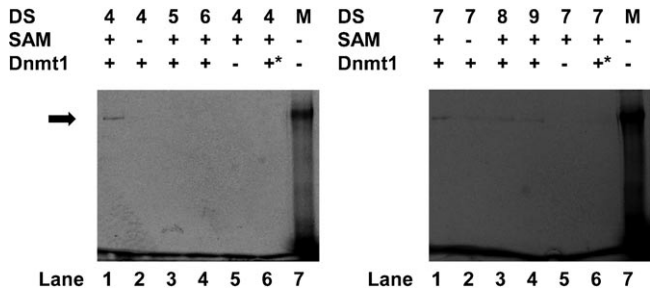

Figure 7. EMSA : Fluorescence detection of DNA–enzyme complexes. The arrow indicates DNA that is bound to the enzyme. M = molecular weight marker, * indicates that Dnmt1 was inactivated before incubation.

tected even for **DS1**, which contains dC^F inhibitor and a natural phosphodiester backbone. **DS4** in contrast provided a clearly visible new band, but only if the SAM cofactor was added. As expected, **DS5** and **DS6** show no detectable adduct with Dnmt1 because of the lack of the dC^F inhibitor. Surprisingly all fully phosphorothioated double strands **DS7**–**9**, however, form stable complexes with the human methyltransferase even if the inhibitor dC^F is not present in the duplex. In this case the complex is also formed in the absence of SAM although the complex band is weaker. We suppose that Dnmt1 forms a covalent bond with ODNs that contain dC^F and noncovalent complexes with phosphorothioated double strands that have no dC^F incorporated. This inhibition model is supported by the fact that the fluorescent bands for **DS8** and **9** as well as for **DS7** without the addition of SAM (lanes 2, 3 and 4) vanish when the reaction mixture was heated for 10 min at 95 °C before gel analysis. In this case, only the complex of **DS7** with Dnmt1 remains visible (data not shown).

To investigate this effect in more detail, we repeated the experiment and analyzed the crosslinking behavior of the double strands **DS1**, **4**, and **7** directly on the same gel. As depicted in Figure 8 A, the inhibitory effect of the duplex **DS1**, which contains the inhibitor and a full phosphodiester backbone is not visible. Even if we increase the concentration of this duplex to 3.3 equivalents, the crosslink is only faintly visible (Figure 8 B).

The crosslinks that were obtained with the phosphorothioate duplexes **DS4** and **DS7** are more visible; this indicates that the phosphorothioates strongly contribute to the inhibitory effect.

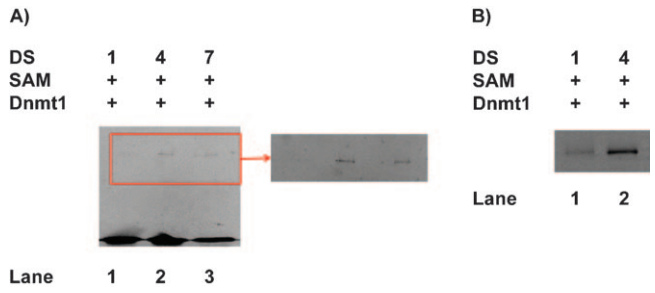

Figure 8. EMSA: A) Comparison of the fluorescent DNA–enzyme complexes of **DS1**, **4**, and **7**. B) Reaction of 25.0 pmol **DS1** and **DS4** with 7.5 pmol Dnmt1.

Restriction protection assay with Dnmt1

To prove that the amount of phosphorothioation is influencing the inhibition of Dnmt1, we used an activity assay that was described by Sowers,[46] which was also recently used by us to analyze dumbbell inhibitors (Figure 9). Dnmt1 was incubated

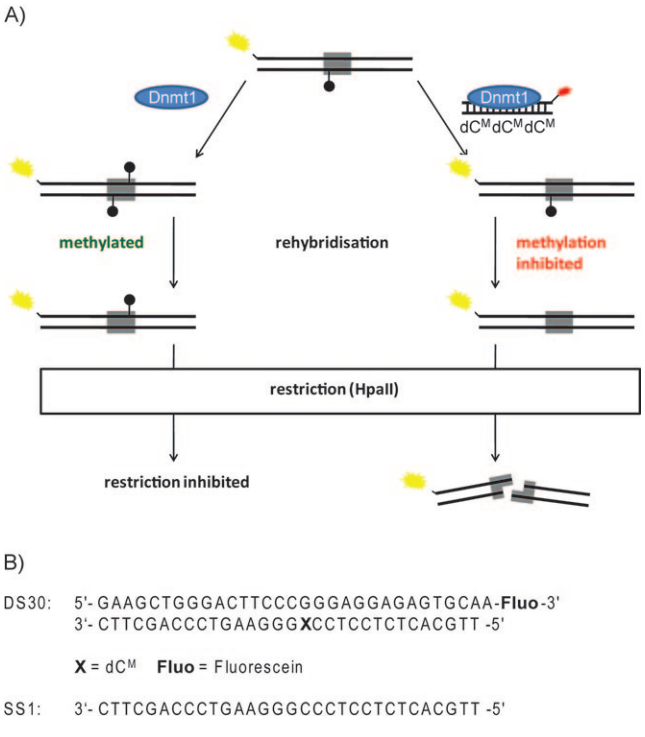

Figure 9. A) Restriction protection assay for Dnmt1. B) DNA that was applied in the assay besides **DS1**–**9**.

with the double-stranded oligonucleotide to be analyzed prior to the assay for 100 min at 37 °C. Then a fluorescently labeled double-stranded 30-mer ODN (**DS30**) that contained a hemimethylated recognition sequence for Dnmt1 was added. This mixture was incubated for another 4 h at 37 °C, and then the reaction was stopped by heating the mixture for 20 min at 60 °C to denature the enzyme.

A large excess of a nonmethylated single-stranded oligonucleotide (SS1) that was complementary to the fluorescently marked substrate DNA strand was added, and the DNA was rehybridized by heating the mixture to 90 °C for 5 min, followed by slowly cooling to room temperature. The reaction was treated with the restriction enzyme HpaII, which cuts only non-methylated recognition sequence. The reaction was finally analyzed by denaturing PAGE. The data are depicted in Figure 10.

All data should be compared to lane 10, where Dnmt1 has methylated DS30, which protects it from restriction (upper band). The lower fluorescent band shows unmethylated, and therefore cut DNA. Control experiments show that HpaII cuts **DS30** without previous methyltransferase treatment (lane 11). The active inhibitor **DS7** in the absence of Dnmt1 does not interfere with the restriction enzyme (lane 12) because here a full cut is observed. For the upper image, the gel was exposed

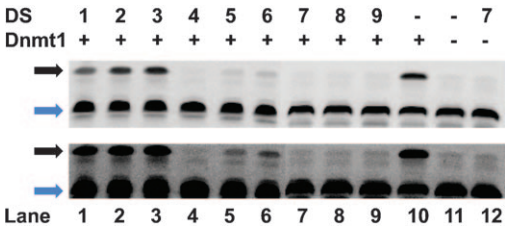

Figure 10. Fluorescence detection of the 30-mer DNA strand. Exposure time: top: 1 s; bottom: 5 s. The black arrow indicates uncut 30-mer, the blue arrow cut 30-mer.

for fluorescence detection for 1 s, for the lower image for 5 s. **DS1** shows a very weak inhibitory effect, which is in agreement with the shift data. In comparison to lane 2, which contains **DS2** in the assay, only a small decrease of the uncut band is observed for **DS1**; this shows the small inhibitory effect of dC^F. **DS2** and **DS3** also show no inhibition of Dnmt1 (lanes 2 and 3). Modification of the phosphodiester backbone in one strand of the duplex by phosphorothioates, however, nearly abolishes the methylation reaction (lanes 4, 5, and 6, top). Only very faint bands could be detected after addition of **DS5** and **DS6**, although **DS5** is slightly more active, possibly because it contains the hemimethylated Dnmt1 recognition sequence (lanes 5 and 6, bottom). The duplex **DS4**, which contains the inhibitor dC^F and one fully phosphorothioated strand shows full inhibition of Dnmt1 (lane 4, bottom). It is clear that the combination of dC^F with phosphorothioates is responsible for the rather strong effect. In addition, and again in agreement with the gel-shift data, the double-stranded **DS7**, in which all phosphodiesters were replaced by phosphorothioates also fully inhibits the enzyme Dnmt1. The data in Figure 10 lanes 7, 8 and 9 show that phosphorothioates alone even in the absence of any suicide inhibitor are able to deactivate Dnmt1.

Conclusions

The bacterial methyltransferase M.HhaI is often used as a model enzyme for the human Dnmt1. It is easier to handle, smaller in size, and more affordable.[47,48] We investigated how small double-stranded oligonucleotides that contain the suicide inhibitor dC^F inhibit both methyltransferases M.HhaI and Dnmt1 when a dC^F is placed opposite a hemimethylated recognition sequence. We studied how a phosphorothioate backbone modification affects the reaction, because these modifications are often used to allow in vivo studies with oligonucleotides. The results show that oligodeoxynucleotides that contain dC^F and a natural phosphodiester backbone are perfect inhibitors for M.HhaI. If the phosphodiesters in one strand are replaced by phosphorothioates this does not change the situation. M.HhaI is perfectly inhibited in the presence of dC^F and not inhibited in its absence. Full replacement of the phosphodiesters, however, counteracts the inhibitory effect of dC^F. In contrast, Dnmt1 is only weakly inhibited by DNA that contains dC^F. Here, however, the phosphorothioates have an astonishingly strong effect. The more phosphodiesters are replaced by phosphorothioates, the better is the inhibitory effect of the duplexes. Even duplexes that contain only phosphorothioates and no dC^F inhibitors are able to fully block Dnmt1. Phosphorothioates are better Dnmt1 inhibitors than dC^F. This result now paves the way for the design of readily available small double-stranded oligonucleotides that are able to inhibit Dnmt1 in vivo.

Experimental Section

Materials: All enzymes, buffers and λ DNA were purchased from New England Biolabs, (Frankfurt am Main, Germany).

Oligonucleotides: Oligonucleotides with a phosphate backbone were synthesized on an Amersham Oligoplot 900 system. Phosphoramidites were acquired from Glen Research GmbH (Sterling, USA). Phosphoramidites and activators were solved in acetonitrile from Riedel de Haen (water content <0.1 ppm), and acetonitrile from Roth was used for all the other reagents (water content <0.3 ppm). The synthesis of the oligonucleotides was accomplished according to the standard protocols of the manufacturer. For the modified nucleotides the coupling time was doubled. Phosphorothioate-modified oligonucleotides were purchased from Metabion (Martinsried, Germany). The synthesized oligonucleotides were separated from the carrier with EtOH/aq NH_3 (3:1). The oligonucleotide concentrations were measured via the absorption coefficients of the nucleobases and the modifications with a nanoDrop3000 device.

HPLC conditions: Analytical HPLCs were performed on a Waters system by using 3 μm C_{18}-reversed phase Nucleodur columns from Machery–Nagel. Eluting buffers were buffer A (0.1 M TEAA in H_2O) and buffer B (0.1 M TEAA in H_2O/MeCN 2:8). The gradient was 0–45% B in 45 min with a flow of 0.5 mL min^{-1}. Preparative HPLC was also performed on a Waters system by using Nucleodur columns (C18ec, 250×10 mm, 5 μm particle size) C_{18}-reversed phase from Machery-Nagel. The gradient was 0–45% B in 45 min with a flow of 5 mL min^{-1}. The elution was always monitored at 260 nm and 545 nm.

Melting points of the oligonucleotides: Melting points of the oligonucleotides were measured on a Varian Cary 100 Bio with temperature controller, transport unit and MultiCellBlock. The temperature gradient was 0.5 °C min^{-1} or 1.0 °C min^{-1}, respectively. Per measurement 5 cooling-down curves (85→12 °C) and 5 heating curves (0→85 °C) at 260 nm and 320 nm were recorded. The measurement of the temperature occurred in a reference cuvette. To prevent a film from forming over the cuvettes at low temperatures, the sample compartment was purged with N_2 during the measurement. Cuvettes of Helma were used with 4 mm inner diameter and 10 mm optical path. The concentration of the oligonucleotides was 3 μM with 150 mM NaCl and 10 mM Tris–HCl (pH 7.4). The solutions in the cuvettes were overlaid with dimethylpolysiloxan to avoid vaporization of the samples. The analysis of the measurements was accomplished by using Microcal Origin (Northampton, USA). Therefore, the curves at 260 nm were averaged, and the averaged background measurement at 320 nm was subtracted. The thus-generated curve was approximated with a 9th-order polynome, the zero point of the second derivation demonstrated the corresponding melting point (Figure 11).

Mass spectrometry: MALDI-TOF mass spectra of the oligonucleotides were recorded on a Bruker Autoflex II mass spectrometer by using 3-hydroxypicolinic acid as a matrix substance. The measurement was arranged in the negative polarity mode (Table 1).

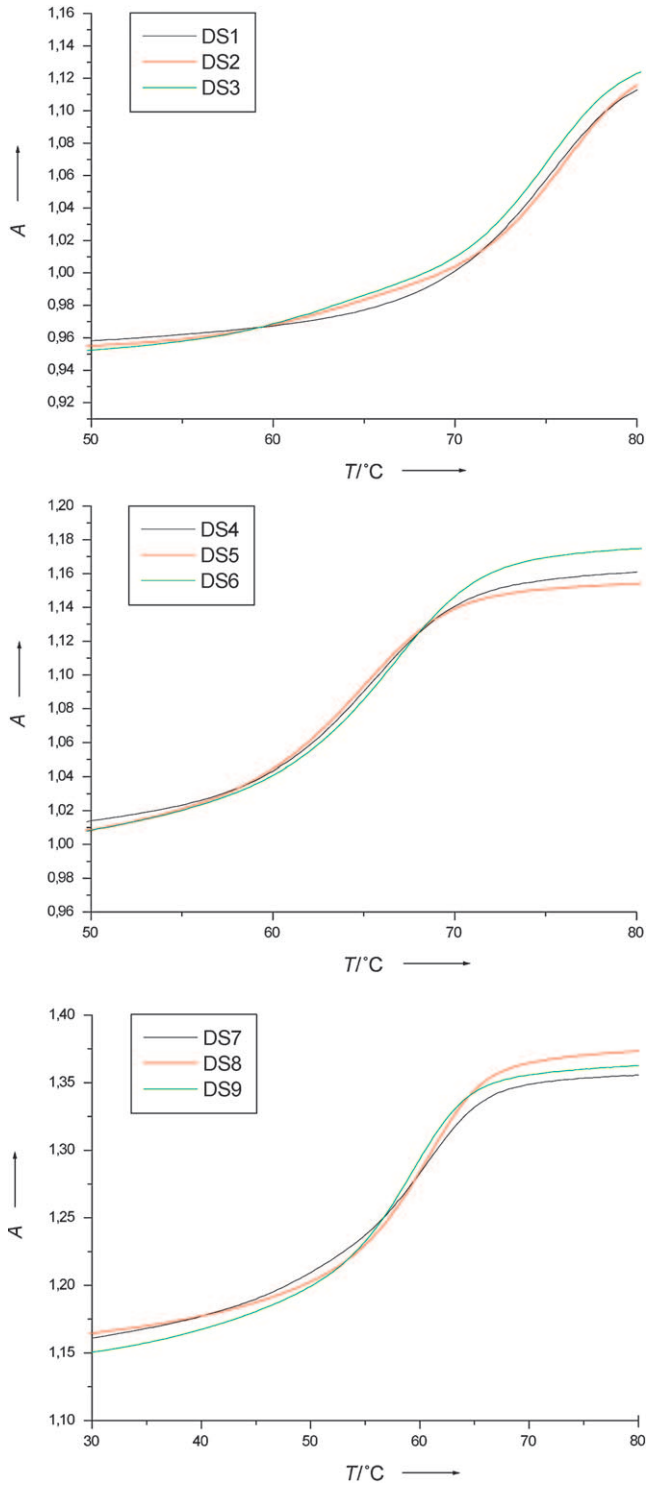

Figure 11. Melting curves: Average melting point (**DS1**–**3**): 75 °C, (**DS4**–**6**): 64 °C, (**DS7**–**9**): 57 °C.

Electrophoretic mobility shift assays: M.HhaI: DNA (3.2 pmol) was incubated with protein (50 U, 13.4 pmol) and SAM (80 μM) in M.HhaI reaction buffer in a total volume of 10 μL for 2 h at 37 °C. The reaction mixture was heated to 95 °C for 5 min and then analyzed by SDS PAGE (12%, 12 mA). Dnmt1: DNA (4.0 pmol) was incubated with protein (10 U, 12.9 pmol) and SAM (160 μM) in Dnmt1 reaction buffer in a total volume of 10 μL for 2 h at 37 °C.

Table 1. Calculated and measured masses of ODNs.

ODN	*M* (calcd)	*M* (found)	ODN	*M* (calcd)	*M* (found)
1	6139.2	6140.7	2	6428.4	6430.6
3	6085.3	6085.9	4	6374.5	6374.1
5	5460.0	5459.9	6	5733.1	5733.8
7	5417.0	5419.2	8	5691.1	5692.7

The reaction mixture was heated to 95 °C for 5 min and then analyzed by SDS PAGE (6%, 12 mA).

Restriction protection assays: M.HhaI: **DS1**, **4**, **7** (4.0, 8.0 and 16.0 pmol) resp. **DS2**, **3**, **5**, **6**, **8**, **9** (16.0 pmol) were incubated with M.HhaI (25 U, 6.7 pmol) and SAM (80 μM) in M.HhaI reaction buffer in a total reaction volume of 10 μL for 75 min at 37 °C. λ DNA (0.5 μg) was added, and the reaction was incubated for another 4 h and then stopped by heating at 65 °C for 15 min. *HhaI Mix* (40 μL) was added (*HhaI Mix*: 10 U HhaI, 100 μg mL^{-1} BSA, 1 mM $MgCl_2$, 1×NEBuffer 4) and the reaction was continued for 30 min at 37 °C. The reaction mixture was analyzed by denaturing PAGE (6%, 12 mA).

Dnmt1: Inhibitor DNA (7.2 pmol) was incubated with Dnmt1 (12 U, 8.8 pmol), SAM (1.6 mM) and BSA (100 μg mL^{-1}) in Dnmt1 reaction buffer in a total reaction volume of 25 μL at 37 °C for 100 min. Then DS30 (10 pmol) and SAM (0.5 μL, 32 mM) were added and the mixture was incubated for another 4 h. The reaction was stopped by heating to 60 °C for 20 min. A large excess of SS1 (25 nmol) was added, and DNA was rehybridized by heating to 95 °C for 5 min followed by slowly cooling to room temperature. Afterwards the reaction mixture was treated with a gel filtration column (10 mM TRIS, pH 7.4, Bio-Rad). Then HpaII (20 U) and NEBuffer 1 (5.8 μL) were added, and the mixture was incubated for 2 h at 37 °C. After desalting on a membrane filter (size of the pores 0.025 μm, Millipore) for at least 30 min, the reaction was completely lyophilized. The residue was taken up in water (10 μL) and analyzed by denaturing PAGE (20%, 12 mA).

PAGE analysis: Samples were analyzed on denaturing polyacrylamide (PAA) gels or SDS gels, respectively (83 mm (w)×82 mm (h)×0.75 mm (d)). Samples on native and denaturing PAA gels were mixed with loading buffer A (50% glycerin (*v*/*v*), 0.2% SDS (*w*/*v*), 0.05% xylene cyanol (*w*/*v*)) and electrophoresed in TBE buffer (1×) at 12 mA for 45–60 min. Samples on SDS gels were mixed with loading buffer B (62.5 mM TRIS, 4% SDS (*w*/*v*), 20% glycerine (*v*/*v*), 5% β-mercaptoethanol (*w*/*v*)) and electrophoresed in TRIS/glycine/SDS buffer (1×) at 12 mA for 45–90 min. The gels were visualized under a LAS3000-Imager (Raytest, Straubenhardt, Germany) and photographed, then they were dyed with Coomassie Blue.

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Keywords: DNA methylation · fluorodeoxycytidine · gene expression · methyltransferases · phosphorothioates

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