DNA Methylation

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Synthesis of DNA Dumbbell Based Inhibitors for the Human DNA Methyltransferase Dnmt1**

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DNA methyltransferases convert deoxycytidine (dC) nucleobases in DNA into 5-methyldeoxycytidines (dC^{Me}) using the cofactor *S*-adenosylmethionine (SAM) as the methyl group donor. Methylation of the canonical dC base, particularly in gene promoter regions, induces complex processes, which finally lead to the silencing of the corresponding gene.[1–4] This epigenetic gene silencing is of paramount importance for cellular differentiation. Altered methylation patterns and corresponding changes in gene expression are found in practically all tumor cells.[5–7] The major DNA methyltransferase Dnmt1 is a 183-kDa-large protein that preferentially methylates dC bases in hemimethylated d(CpG) sequences after DNA replication.[8–11]

Dnmt1 was shown to be essential for the maintenance of DNA methylation in mouse and human cells.[12–14] Efficient inhibition of Dnmt1 therefore offers the possibility of interfering with the methylation process, which may allow control of the epigenetic programming/reprogramming of cells. Today, however, only a very small number of molecules are known that interfere with the epigenetic mechanisms.[15–20] Among the best studied compounds are 5-azadC (dC^N),[21] 5-azaC,[22] zebularine,[23] and 5-fluorodC,[24] which if added to cells are converted to the corresponding triphosphates. These are typically accepted by DNA polymerases and incorporated into the cellular genome, where they act as suicide inhibitors for DNA methyltransferases such as Dnmt1 by forming a covalent bond between the C6 position of the inhibitory base and a catalytically essential Cys residue.[22,23,25,26] Because these compounds are incorporated into the genome of the treated cells, all of these compounds are cytotoxic and persistent nucleoprotein complexes are formed in the genome.[27,28] Alternative siRNA-based attempts to fully deplete the Dnmt1 activity also failed owing to an insufficient silencing effect.[29] To create molecules that are able to fully inhibit Dnmt1 without being integrated into the host genome, we have investigated small DNA structures (Scheme 1), in which the best known, but very sensitive, suicide inhibitor for methyltransferases, 5-azadC, was incorporated to covalently trap Dnmt1. Inside DNA, the fragile nucleotide is protected and stabilized.[30] Because the DNA structures have to function inside living cells, we prepared small circular DNA dumbbells, which should have a significantly longer lifetime in serum (see the Supporting Information).[31,32] However, the sensitivity of the inhibitor dC^N makes its chemical incorporation into DNA dumbbells intractable. We therefore developed a novel chemoenzymatic approach for the preparation of the desired highly functionalized dumbbell structures.[33]

Scheme 1. Inactivation of the human methyltransferase Dnmt1 by formation of a covalent linkage between the inhibitory dumbbell and the enzyme in the presence of SAM. C^N = 5-azadC, C-Me = 5-methyldC, Fl = Cy3 fluorescence label.

The synthesis and characterization of the dC^N-containing dumbbells is depicted in Scheme 2. The starting point is a synthetic 34-mer DNA strand (see the legend of Scheme 2) with a central $d(GpC^{Me})_3$ segment (in italics) and a dT_4 loop region at each end, which is partially complementary to the DNA flanking the $d(C^{Me}pG)_3$ unit. The special sequence with the $d(C^{Me}pG)_3$ unit was chosen to target specifically Dnmt1, which binds preferentially to such hemimethylated sites.[8,11,34] This design forces the loop structures to fold back until both ends are aligned just opposite the $d(C^{Me}pG)_3$ segment (Scheme 2a). A 5′-phosphate was synthetically attached to the DNA structure to allow later ligation. Next, we enzymatically extended the loop structure, starting from the 3′-end, after addition of a mixture of dGTP and dC^NTP, using the exonuclease-lacking Klenow fragment (KF^-) as the DNA polymerase. Because of the presence of the 5′-phosphate we could finally ligate the properly filled-in DNA structures with T4 ligase.

Scheme 2b shows the gel electrophoretic and mass spectrometric analysis of the reaction sequence. The filled-in extended DNA structure, as the first reaction product (product 1, middle), has slightly reduced mobility in the gel. Ligation produces a new DNA compound (product 2, right) which has mobility intermediate to that of product 1 and of the DNA starting material. In addition, the high-resolution

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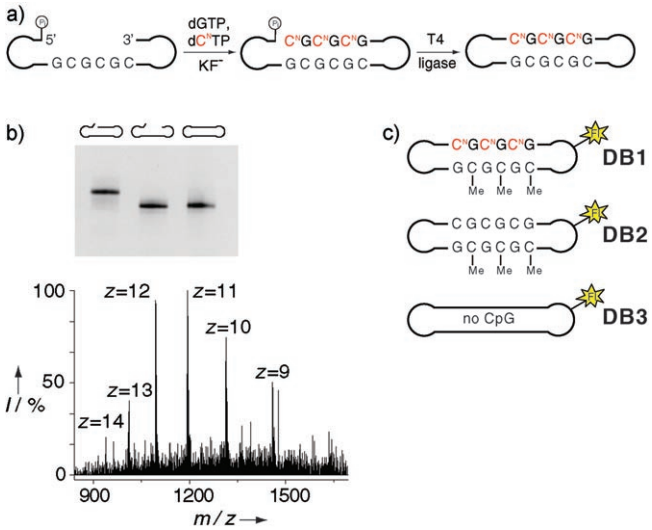

Scheme 2. Synthesis and characterization of the dumbbell inhibitors. a) Synthesis of the dumbbells by fill-in DNA polymerization with the Klenow fragment (*exo*-) and ligation with T4 ligase. b) Polyacrylamide gel electrophoretic analysis of the reaction sequence (20%, 12 mA; top) and mass spectrum (ESI-FTICR; bottom) of the dumbbell **DB1**. c) The three dumbbells prepared for this study. DNA sequence for **DB1**: 5′-d(pAGAGCTTTTGCTCTC^{Me}GC^{Me}GC^{Me}GACTCCT^{Cy3}TTTGGAGT)-3.

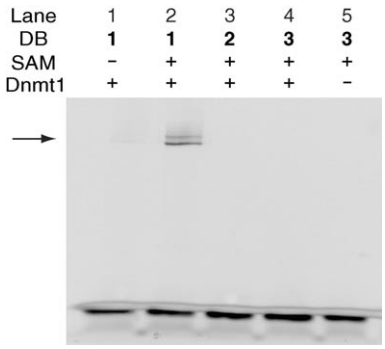

Figure 1. EMSA of DNA dumbbells and Dnmt1. The arrow indicates DNA covalently bound to the enzyme. A 4-pmol sample of DNA was incubated with 4 pmol (5.5 U) of Dnmt1 for 2 h at 37 °C in the presence of 160 μM SAM in 50 mM Tris-HCl, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol at pH 7.8. Analysis by SDS PAGE (6%, 120 V). Tris = tris(hydroxymethyl)aminomethane.

electrospray mass spectrum showed signals corresponding to the molecular weight expected for product 2. Excellent agreement between the expected molecular weight for, for example, m/z $(z,12) = 1093.1786$ and the calculated value for m/z $(z,12) = 1093.1843$ (for **DB1**) together with the clean polyacrylamide gel proved the successful preparation of the desired dumbbell structures.

Scheme 2c shows the three DNA dumbbells prepared for this study. Both **DB1** and **DB2** contain the hemimethylated central segment that is the natural substrate for Dnmt1, but only **DB1** contains the dC^N inhibitor. **DB3** contains no d(C^{Me}pG)$_3$ units and serves as a pure control dumbbell. All three dumbbells feature a Cy3 fluorescence label (Fl) at one of the dT bases in the loops to allow detection.

To study the binding of the dumbbell inhibitors to Dnmt1, we developed an electrophoretic mobility shift assay (EMSA). Each of the dumbbells **DB1**–**DB3** (4 pmol) was added to a solution of 160 mM SAM and 4 pmol (5.5 U) of Dnmt1 at pH 7.8. After 2 h of incubation at 37 °C the solutions were loaded onto sodium dodecylsulfate (SDS) polyacrylamide gel and analyzed by fluorescence detection.

The study clearly shows that a complex is formed between **DB1** and Dnmt1 (Figure 1). No complexes between the control dumbbells **DB2**/**DB3** and Dnmt1 were observed, showing that Dnmt1 binds selectively to duplex structures with hemimethylated sites to form a covalent complex with the additionally present dC^N. We explain the fact that two small bands are formed by the complex nature of the enzyme Dnmt1. Most important, however, is the observation that no complex is formed in the absence of the inhibitor dC^N even when a hemimethylated site is present. We also see a very faint band when no cofactor is present (lane 1). This is in accord with literature reporting that dC^N-containing DNA inhibits Dnmt1 even in the absence of SAM.[35,36]

The presence of excess hemimethylated DNA did not change the result (see Figure S3 in the Supporting Information). Furthermore, addition of the non-fluorescent-labeled **DB1** dumbbell DNA (termed **DB4**) to the enzyme–DNA complex did not reduce the fluorescence signal, proving covalent linkage of the inhibitory dumbbell (see Figure S4 in the Supporting Information).

To investigate the inhibitory effect of our new DNA dumbbells on Dnmt1, we used an activity assay described recently by Sowers and co-workers[37] (Scheme 3a). A normal DNA duplex with a hemimethylated recognition site for the dC^{Me}-inhibited restriction enzyme HpaII and a fluorescence tag is added to a solution containing Dnmt1 and SAM. If Dnmt1 is active, methylation occurs to form a fully methylated site. Dehybridization followed by rehybridization with unmethylated counterstrands gives in this case a hemimethylated duplex, which cannot be cleaved by HpaII. Inactive Dnmt1, in contrast, cannot methylate, which finally provides a fully unmethylated restriction site, which will be cleaved by HpaII.

The experiment shows that in the presence of Dnmt1, methylation occurs, which fully inhibits HpaII (Scheme 3b, lane 5). In the absence of Dnmt1 no methylation occurs, allowing HpaII to cleave (lane 4). In the presence of the dumbbells **DB2** and **DB3**, Dnmt1 is active and induces methylation, which blocks HpaII (lanes 2, 3; analogous to lane 5: no dumbbell). The dumbbell **DB1**, in contrast, is able to fully inhibit methylation, which allows HpaII to cleave the DNA (lane 1). The results show that the dC^N-containing DNA dumbbell **DB1** binds to Dnmt1 and blocks the methylation reaction. Roughly stoichiometric amounts of **DB1** are needed to fully inhibit the enzyme, proving the suicide mode of activity. Besides Dnmt1 we also investigated various bacterial methyltransferases such as M.SssI and found that **DB1** binds and inhibits these enzymes as expected (see the Figures S5 and S6 in the Supporting Information).[32]

To investigate these compounds in mammalian cells, we transfected human HTC116 tumor cells and, as a model,

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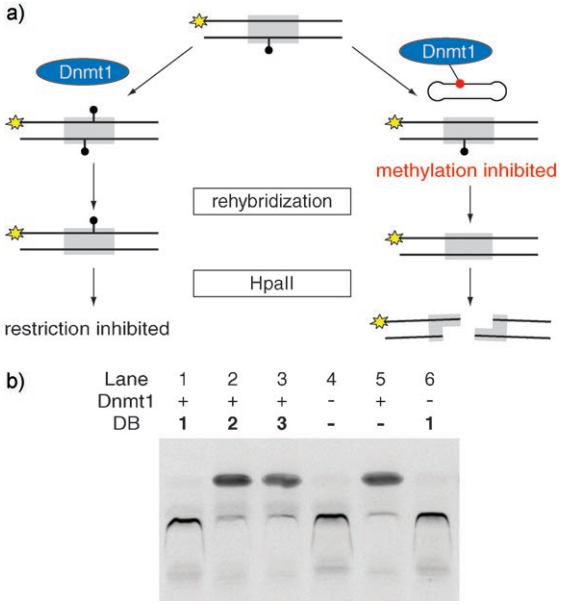

Scheme 3. a) Representation of the restriction protection assay for Dnmt1. After incubation with the inhibitor, Dnmt1 is unable to methylate a hemimethylated 30-mer DNA strand (**DS1**, sequence see Table S1 in the Supporting Information) and thereby protects it from restriction digestion by HpaII. ●: 5-methyl-dC; gray area: HpaII recognition site (C′CGG); yellow star: fluorescein label. b) Analysis of the restriction protection assay by denaturing PAGE (20%, 12 mA).

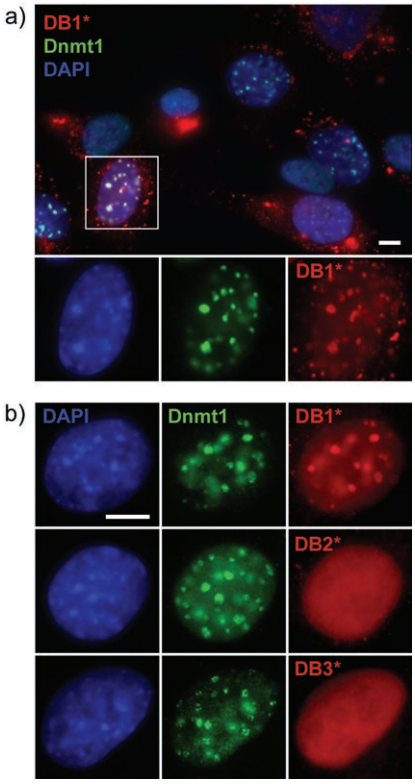

Figure 2. Treatment of C2C12 myoblast cells with dumbbell constructs (red). After formaldehyde fixation of the cells, endogenous Dnmt1 was detected with specific antibodies (green) and DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI, blue). White scale bars: 5 μm. a) Transfection with **DB1*** using HiPerFect transfection reagent. The inset shows an enlarged view of the cell nucleus. b) C2C12 cell nuclei 1 h after microinjection with dumbbell constructs. **DB1*** colocalizes with Dnmt1 at replication sites, whereas **DB2*** and **DB3*** show a diffuse distribution in the cell nucleas.

mouse C2C12 myoblast cells with our dumbbell constructs using Transfectin and HiPerFect as the delivery agent, respectively. By fluorescence microscopy we observed that the dumbbells entered efficiently into cell nuclei. We observed no toxicity on mouse cells in our initial experiments. We immunostained endogenous Dnmt1 in C2C12 cells 16 h after transfection with **DB1*** (a variant of DB1 with two Cy3 labels, see Table S1 in the Supporting Information) and found the active inhibitor in nuclei of S phase cells colocalizing with Dnmt1 accumulated at active replication sites (Figure 2a). When microinjecting the dumbbell constructs directly into the nucleus, we observed colocalization of **DB1*** with Dnmt1 at replication sites of S phase cells already within 1 h. In contrast, the non-dC^N-containing controls **DB2*** and **DB3*** showed no such association (Figure 2b).

Transfected into HCT116 cancer cells, **DB1** effected a significant reduction of cell proliferation (see Figure S8 in the Supporting Information). These results indicate that Dnmt1 recognizes and stably interacts with the dumbbell **DB1** in living cells.

Our compounds are therefore among the first chemical entities able to inhibit Dnmt1, which is one of the major enzymes involved in epigenetic control of gene expression. The ability of these compounds to enter the nucleus of living cells and to bind Dnmt1 offers exciting new possibilities for the regulation of the methylation maintenance reaction.

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