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An oligodeoxyribonucleotide containing 5-formyl-2'-deoxycytidine (fC) at the CpG site forms a covalent complex with DNA cytosine-5 methyltransferases (DNMTs)

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ABSTRACT

5-Methylcytosine (mC) is known to induce epigenetic changes. Ten-eleven translocation (TET) enzymes produce the further oxidized 5-substituted cytosine derivatives, 5-formylcytosine (fC) and 5-carboxylcytosine (caC). However, their roles are unclear thus far. Here, we synthesized oligodeoxyribonucleotides (ODNs) containing 5-formyl-2'-deoxycytidine and examined their interactions with DNA cytosine-5 methyltransferase (DNMT). We found that the ODN sequence containing fCpG formed a covalent complex with both bacterial and mouse recombinant DNMTs in the absence of any cofactors. The covalent bonding with DNMT suggests that the fCpG sequence in DNA may play a role in epigenetic regulation.

Methylation at the C5 position of cytosine in a CpG dinucleotide is catalyzed by DNA cytosine-5 methyltransferase (DNMT) to give 5-methylCpG (mCpG), using *S*-adenosyl-L-methionine (SAM) as the methyl group donor (Figure 1A). This modification has a profound effect on gene expression, particularly in gene promoter regions.^{1,2} One characteristic of tumor cells is their unusual DNA methylation patterns. There are a number of repetitive DNA sequences, and some genes are hypermethylated, leading to silencing of the gene.³⁻⁵ Demethylation of mCpG has been extensively studied, and ten-eleven translocation 1 (TET1) has been found to oxidize the methyl group in mC to give 5-hydroxymethylcytosine (hmC) as a first step in the demethylation pathway.⁶⁻¹⁰ In subsequent studies, it was shown that hmC is also a substrate of TET1, 2 and 3, with the reaction of hmC producing the further oxidized 5-substituted cytosine derivatives, 5-formylcytosine (fC) and 5-carboxylcytosine (caC).¹¹⁻¹⁵ Because fC and caC are good substrates of thymine-DNA glycosylase (TDG), the bases can be converted to an appropriate form to trigger base-excision repair (BER) to return to CpG, the demethylated form (Figure 1C).¹⁶ It was discovered that DNMT can catalyze the direct dehydroxymethylation of hmC¹⁷ and decarboxylation of caC to give unmodified cytosine in DNA.¹⁸ Thus, hmC, fC and caC are primarily believed to be intermediates in the demethylation pathway.¹⁹ However, hmC is believed to play an important role in epigenetic programming of the genome and regulation of tissue-specific gene expression and, therefore, could add another layer

of complexity to the intricate network of epigenetic regulation.^{20,21} Recently, Bachman *et al.* reported that fC levels in mammalian DNA are not correlated with those of its precursors, mC and hmC, or its metabolite caC.²² Many proteins, including transcriptional regulators, DNA repair factors and chromatin regulators, have been known to preferentially bind to ODNs containing fC compared to those containing only mC or hmC.^{23,24} X-ray crystallography of a duplex ODN containing (fCpG)₃ revealed helical underwinding, and these conformational changes may directly control the recruitment of fC reading proteins.²⁵ Therefore, fC may have functional roles in DNA that go beyond being simply a demethylation intermediate. Here, we found that an ODN containing fC in a CpG sequence reacts with DNMTs through the formation of a covalent bond, similarly to ODNs containing 5-aza-2'-deoxycytidine (d^NC),²⁶⁻²⁸ 5-fluoro-2'-deoxycytidine (d^FC)^{29,30} and zebularine.³¹ They act as suicide inhibitors for DNMTs by covalent bond formation between the C6-position of the base moieties and a catalytically essential Cys residue same as natural methylation mechanism (Figure 1B) after incorporation at the CpG site in DNA by DNA polymerases.¹⁷⁻¹⁹

Introduction of a formyl group at the C5-position of cytosine moiety gives an α,β -unsaturated aldehyde, and the C6-position of the cytosine become more electron-deficient than that of cytosine itself. Therefore, we hypothesized that a DNMT would react with an ODN having fCpG sequences similar to d^NC, d^FC and zebularine (Figure 2A).

Results and Discussion

Figure 1. A) Methylation by DNA cytosine-5 methyltransferases (DNMTs) with SAM as a cofactor. B) Plausible mechanism of cytosine methylation by DNMTs. C) Methylation of cytosine and demethylation pathways.¹⁷⁻¹⁹ D) Sequences of synthesized ODNs containing d^FC, fC and 5-methyl-2'-deoxycytidine (mC).

First, we calculated electron density of the C6-position by Mulliken population analysis (Figure 3). Cytosine revealed at +0.11, and ^FC and ^NC are more electron-negative as +0.15 and +0.29, respectively. The formyl group also decreased the electron density at the C6-position as +0.16 quite similar to that of ^FC. Therefore, fC could react with the Cys residue at the active site of DNMT from the point of the electron negativity at the C6-position. On the other hand, a size of a substituent at the C5-position of cytosine in the CpG sequence is known to be important for recognition by DNMT. In a 5-halogenocytosine series, only the 5-fluorocytosine (^FC) in the CpG sequence showed to react with DNMTs in the presence of SAM as shown in Figure 2B. Although a size of the 5-chloro and 5-bromo substituents is similar to that of a methyl group, the dsODNs containing these substituents were not recognized as inhibitors or

Figure 2. Plausible complex structures of A) fC and B) ^FC with DNMTs.

Figure 3. Mulliken population of C, ^FC, ^NC and fC at the C6-position calculated by Jaguar 7.7.

substrates by DNMTs, due might be sterical crash with the Pro in the active site.³² A size of the formyl group is sterically less hindered and planar by the sp² carbon. Therefore, we synthesized ODN containing fC (ODN-fC) for the examination of the reaction with DNMTs.

The ODNs containing fC (ODN-fC) were prepared using a method we previously described during studies of DNA oxidative lesions.³³ ODN-^FC was used for control experiments as a covalent bond-forming ODN, and complementary ODN-G and ODN-M were also synthesized. To investigate the interaction of ODN-fC with DNMT, we first studied a bacterial DNMT, M.HpaII, from *Haemophilus parainfluenzae*. M.HpaII typically methylates the internal 2'-deoxycytidine in the target sequence 5'-CCGG-3', but in our studies, fC was substituted in place of the internal 2'-deoxycytidine (ODN-fC). Annealing ODN-G with 5'-³²P-labeled ODN-fC or ODN-^FC gave the double-stranded (ds)-fC:G and ds-^FC:G, respectively (Figure 1D). Each ds-ODN was incubated with 4 equiv. of M.HpaII at 37 °C for 5-60 min. The reactions were quenched with loading buffer containing 7 M urea, and the reaction mixture was analyzed by denaturing polyacrylamide gel electrophoresis (PAGE). The reaction with ds-fC:G showed rapid and efficient formation of an ODN-M.HpaII complex, giving greater than 90% yield after 5 min reaction time (Figure 4A, lane 1). The formyl group was allowed to recognize by the DNMT from our experiments. A formyl group (van Der Waals surface (vdW); 34.1 by Maestro 10 software) is sterically smaller than hydroxymethyl (vdW; 41.4) and carboxyl (vdW; 39.6) groups and a same size as a methyl group (vdW; 33.2) by its sp² orbital and hydrogen substituent. This association rate is comparable to that of a previously reported disulfide-based DNA probe.³⁴ The high binding affinity of the ODN may cause non-sequence-specific binding to DNMTs. To investigate whether formation of the covalent linkage between ODN-fC and M.HpaII is sequence specific or not, we examined ODN-fC*, in which the external dC in 5'-CCGG-3' was replaced with fC (Figure 1D). In this case, M.HpaII did not form a complex with ds-fC*:G/M at all (Figure S1). Therefore, the methyltransferase binds to ds-fC:G with a sequence specific manner. On the other hand, ds-^FC:G showed a band corresponding to a complex with M.HpaII in 40% yield after 60 min reaction time (Figure 4A, lane 8). It was reported that ODNs containing d^NC (decitabine, a clinically approved drug for treatment of myelodysplastic syndrome) in a CpG sequence instead of cytosine had a high rate of a complex formation with

M.HpaII in both the absence and presence of SAM and S-adenosyl-L-homocysteine (SAH).^{27,28} This is important because the SAM/SAH ratio is different in each tissue,³⁵ and a cofactor-independent inhibitor is desired for clinical use. As shown in Figure 4B, ds-^FC:G formed a complex with M.HpaII only in the presence of SAM (lanes 2-4). Moreover, the complex formation of ds-^FC:G occurred in high yield and independent of cofactors such as SAM and SAH (lanes 5-7). Therefore, DNMT binds to ds-^FC:G more efficiently than ds-^FC:M, and this occurs in the presence or absence of any cofactors. We next investigated whether ds-^FC:G forms a covalent complex or not.

Figure 4. Gel shift assay of ds-^FC:G and ds-^FC:M. A) ODN-^FC and ODN-^FC were 5'-³²P labeled and annealed with ODN-G. The reactions were performed with 0.1 μM ds-^FC:G or ds-^FC:M and 240 μM SAM by incubating at 37 °C for 5-60 min with 0.4 μM M.HpaII in 50 mM Tris-HCl (pH 7.5), 10 mM EDTA and 5 mM β-mercaptoethanol, and analyzed by denaturing PAGE (8%, 140 V, 1 h). Lanes 1 and 5; 5 min, lanes 2 and 6; 15 min, lanes 3 and 7; 30 min, lanes 4 and 8; 60 min, B) The reactions were performed with 0.1 μM ds-^FC:G or ds-^FC:M and 240 μM SAM or SAH (or no cofactor) by incubating at 37 °C for 2 h with 0.4 μM M.HpaII in 50 mM Tris-HCl (pH 7.5), 10 mM EDTA and 5 mM β-mercaptoethanol, and analyzed by denaturing PAGE (8%, 140 V, 1 h). Lane 1; control ds-^FC:G, lane 2; ds-^FC:G with SAM, lane 3; ds-^FC:G with SAH, lane 4; ds-^FC:G without SAM and SAH, lane 5; ds-^FC:G with SAM, lane 6; ds-^FC:G with SAH, lane 7; ds-^FC:G without SAM and SAH.

The reversibility of the ODN-M.HpaII complex was studied in the presence of a large excess of non-labeled exchanger ds-^FC:G (Figure S2). The complex formed with SAM or SAH (Figure 4B, lanes 5 and 6) was not disrupted by the addition of 100 equiv. of non-labeled ds-^FC:G after 48 h of incubation, while the ³²P-labeled complex was disrupted ($t_{1/2}$ 56.2 h) in the absence of cofactors (Figure 4B, lane 7 and Figure S2B).

Finally, mouse recombinant Dnmt1³⁶ was studied for complex formation with ds-^FC:M and ds-^FC:M. While ds-^FC:M formed the complex in 19% yield after a 30 min reaction (Figure 5A, lane 4), the rate of complex formation with ds-^FC:M was much faster with over 80% yield after a 30 min reaction (Figure 5A, lane 9). The effect of cofactors on complex formation was also studied as described previously with the bacterial enzyme. Complex formation with ds-^FC:M did not require any cofactors and the product was stable in denaturing PAGE, while the complex with ds-^FC:M required SAM for stable complex formation (Figure S3). Although many types of DNA binding proteins are present in the nuclear extract of MCF-7 cells, only one ³²P-labeled ODN-protein complex band was observed on PAGE when ds-^FC:M was incubated with the extract (Figure 5B), suggesting that ds-^FC:M binds strongly and selectively to Dnmt1 in cells.

In summary, we have synthesized an ODN containing 5-formyl-2'-deoxycytidine (fC) instead of 2'-deoxycytidine in a CpG sequence. fC is known to be an intermediate in the oxidative demethylation pathway of an mCpG sequence. It was found that ds-^FC:G is capable of highly efficient formation of ODN-DNMT

Figure 5. Gel shift assay of ds-^FC:M and ds-^FC:M. A) ODN-^FC and ODN-^FC were 5'-³²P labeled and annealed with ODN-G. The reactions were performed with 0.1 μM ds-^FC:M or ds-^FC:M and 240 μM SAM by incubating at 37 °C for 5-120 min with 0.4 μM mouse recombinant Dnmt1 in 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10 mM NaCl, 5 mM β-mercaptoethanol, 5% glycerol and 20 μg/mL BSA, and analyzed by denaturing PAGE (8%, 140 V, 1 h). Lanes 2 and 7; 5 min, lanes 3 and 8; 15 min, lanes 4 and 9; 30 min, lanes 5 and 10; 60 min, lanes 6 and 11; 120 min. B) EMSA of ds-^FC:M with nuclear extract of MCF-7 cells. The ds-^FC:M (0.2 μM) was incubated with nuclear extract of MCF-7 cells (2 μg/mL) at 37 °C for 120 min and analyzed by PAGE. Lane 1; ds-^FC:M without nuclear extract of MCF-7 cells, lane 2; ds-^FC:M with nuclear extract of MCF-7 cells.

complex with both bacterial DNMT (M.HpaII) and mouse recombinant Dnmt1 in the absence of any cofactors such as SAM and SAH. Therefore, our research suggests that fC in a CpG sequence of DNA may not be just an intermediate in the demethylation pathway, waiting for repair with the TDG/BER system to furnish the demethylation of mCpG to CpG, but may play a role in the DNA methylation/demethylation regulation pathway by specific and efficient complex formation with DNMT.

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Supplementary Material

Supplementary data associated with this article can be found, in the online version.