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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 (fC) 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, 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 (dNC),26-28 5-fluoro-2′-deoxycytidine (dFC)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.32,33

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 dC, dC and zebularine (Figure 2A).
Results and Discussion

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 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, IC was substituted in place of the internal 2′-deoxycytidine (ODN-fC). Annecing 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 dC (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.HhaI in both the absence and presence of SAM and S-adenosyl-L-homocysteine (SAH). This is important because the SAM/SAH ratio is different in each tissue, and a cofactor-independent inhibitor is desired for clinical use. As shown in Figure 4B, ds-5′C-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-5′C-G, 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-5′C-G: A) ODN-IC and ODN-5′C were 5′-32P labeled and annealed with ODN-G. The reactions were performed with 0.1 μM ds-FC-G or ds-fC-G 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). Lane 1; control ds-FC:M, lane 5; ds-fC with SAM, lane 6; ds-fC-G with SAM, lane 7; ds-fC-G with SAM and SAH, lane 8; ds-fC-G without SAM and SAH, lane 9; ds-fC with SAM and SAH, lane 10; 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:M (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:M after 48 h of incubation, while the 5′-32P-labeled complex was disrupted (t1/2 56.2 h) in the absence of cofactors (Figure 4B, lane 7 and Figure S2B).

Finally, mouse recombinant Dmnt1 was studied for complex formation with ds-fC:M and ds-5′C-M. While ds-5′C-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-5′C-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 5′-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 Dmnt1 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 complex with both bacterial DNMT (M.HpaII) and mouse recombinant Dmnt1 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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References and notes


Supplementary Material

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