Bioorganic & Medicinal Chemistry Letters 26 (2016) 5395–5398

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

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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ARTICLE INFO

Article history:
Received 14 September 2016
Revised 11 October 2016
Accepted 13 October 2016
Available online 14 October 2016

Keywords:
Epigenetic
Nucleic acids
5-Formyl-2′-deoxycytidine
Covalent bond
DNA methyltransferase

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 (Fig. 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 (Fig. 1C).[16] It was discovered that DNMT can catalyze the direct dehydroxymethylation of hmC[17] 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 (Fig. 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 reacts with

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http://dx.doi.org/10.1016/j.bmcl.2016.10.040
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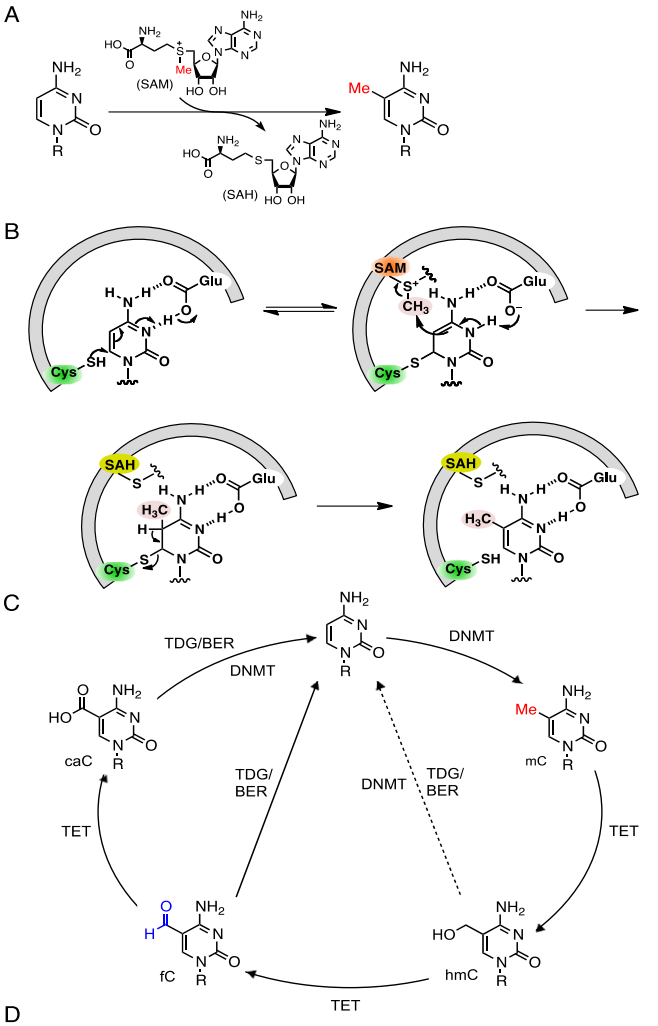

	ODN-G	ODN-M
ds-^{F}C:G	5'-TCTGAT C^{F}CGGTGACAT-3' 3'-AGACTAGG CCACTGTA-5'	ds-^{F}C:M 5'-TCTGAT C^{F}CGGTGACAT-3' 3'-AGACTAGG **mC**CACTGTA-5'
ds-fC:G	5'-TCTGAT C**f**CGGTGACAT-3' 3'-AGACTAGG CCACTGTA-5'	ds-fC:M 5'-TCTGAT C**f**CGGTGACAT-3' 3'-AGACTAGG **mC**CACTGTA-5'
ds-fC*:G	5'-TCTGAT**f**C CGGTGACAT-3' 3'-AGACTA GG CCACTGTA-5'	ds-fC*:M 5'-TCTGAT**f**C CGGTGACAT-3' 3'-AGACTA GG **mC**CACTGTA-5'

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^{F}C, fC and 5-methyl-2′-deoxycytidine (mC).

an ODN having fCpG sequences similar to d^{N}C, d^{F}C and zebularine (Fig. 2A).

Results and discussion

First, we calculated electron density of the C6-position by Mulliken population analysis (Fig. 3). Cytosine revealed at +0.11, and ^{F}C and ^{N}C 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 ^{F}C. 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 (^{F}C) 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 sp^2 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-^{F}C 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′-C**C**GG-3′, but in our studies, fC was substituted in place of the internal 2′-deoxycytidine (ODN-fC). Annealing ODN-G with 5′-^{32}P-labeled ODN-fC or ODN-^{F}C gave the double-stranded (ds)-fC:G and ds-^{F}C:G, respectively (Fig. 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 (Fig. 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 sp^2 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′-**C**CGG-3′ was replaced with fC (Fig. 1D). In this case, M.HpaII did not form a complex with ds-fC*:G/M at all (Fig. S1). Therefore, the methyltransferase binds to ds-fC:G with a sequence specific manner. On the other hand, ds-^{F}C:G showed a band corresponding to a complex with M.HpaII in 40% yield after 60 min reaction time (Fig. 4A, lane 8). It was reported that ODNs containing d^{N}C (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-homocys-

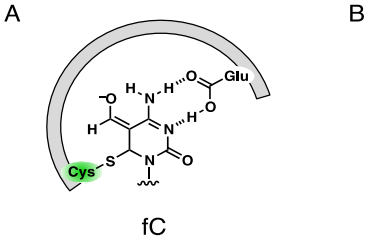

Figure 2. Plausible complex structures of (A) fC and (B) ^{F}C with DNMTs.

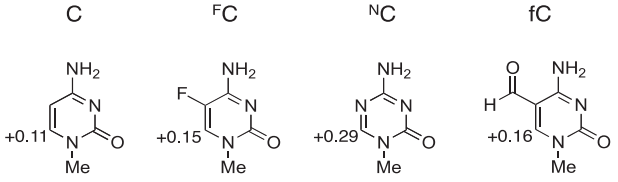

Figure 3. Mulliken population of C, ^{F}C, ^{N}C and fC at the C6-position calculated by Jaguar 7.7.