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Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 17 (2007) 3682–3685

Improvement of base selectivity and binding affinity by controlling hydrogen bonding motifs between nucleobases and isoxanthopterin: Application to the detection of T/C mutation

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Received 25 November 2006; revised 3 April 2007; accepted 13 April 2007
Available online 19 April 2007

Abstract—At an abasic site in an oligo-DNA duplex, isoxanthopterin (**IX**)[†] can bind to thymine (T) and cytosine (C) with strong affinity compared to adenine and guanine, but the base selectivity for T against C is moderate. In order to improve both binding affinity and base selectivity for T against C, a methyl group is introduced to **IX**, which is known as 3-methyl isoxanthopterin (**3-MIX**),[†] by which binding affinity for C is expected to decrease. Indeed, **3-MIX** specifically binds to T more strongly than **IX** and loses its binding affinity for C. The improved binding ability of **3-MIX** for T would be suitable for the practical use in SNP typing related to T.

Interaction between small molecules, i.e., ligands, and DNA duplexes has been paid much attention in various fields such as regulation of gene expression for drug design[1] and DNA-based chemical sensors.[2] Binding behavior between ligands and DNA duplexes has been investigated for molecules such as hydrogen bonding ligands,[3–6] groove binders,[7–9] and intercalators.[10,11] In bio-sensing systems, several SNP (single nucleotide polymorphism) typing methods have been proposed based on the binding of small molecules, i.e., ligands, to oligo-DNA duplexes, because SNPs are responsible for most of the genetic variation related to diseases, drug response, and drug metabolism.[12–14] Methods based on SPR (surface plasmon resonance),[3,4] fluorescence,[5,6] and electrochemical[15–17] have been applied to detect the genetic variation using ligands with hydrogen bonding or intercalating ability to oligo-DNA duplexes. Considering the high throughput detection of the genetic variation, detection of fluorescence would be promising because it can be easily applied to array sensing systems with quick response.[11]

Whereas most of the current methods of detecting SNPs by fluorescence require labeling fluorophores into oligo-DNAs,[18–23] we have recently proposed to use an oligo-DNA containing an apurinic or apyrimidinic site, that is an abasic site or an AP site as a recognition field for target nucleotides, by which fluorescence detection has been realized without labeling fluorophores into oligo-DNAs.[5,6] While naturally occurring AP sites are the most common forms of DNA damages, we intentionally constructed the AP site in an oligo-DNA duplex so as to orient the AP site toward a target nucleobase, by which hydrophobic microenvironments are provided for ligands to recognize nucleotides through a Watson-Crick hydrogen bonding motif. We have investigated a series of fluorescent ligands which have specific hydrogen bonding moieties and are fully complementary to the unpaired nucleobase opposite the AP site in an oligo-DNA duplex.[5,6,24] We have demonstrated that 2-amino-7-methyl-1,8-naphthyridine (AMND),[5,24a] 2-amino-4-oxopteridine (pterin),[6] and vitamin B_2 (riboflavin)[24b,4c] can effectively detect SNPs related to cytosine (C), guanine (G), and thymine (T), respectively. We have also reported that the binding constant for T can be enhanced by using amiloride as a ligand based on the charge–charge interaction between the guanidinium moiety of amiloride and the DNA phosphate backbone,[24d] and that introducing methyl groups to pterin enhances the recognition ability for G due to the

Keywords: AP site; Methyl group; T/C SNP; p53 Gene.
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† **IX** and **3-MIX** was obtained from Sigma–Aldrich Co. (USA) and Enamine Ltd, (Ukraine), respectively.

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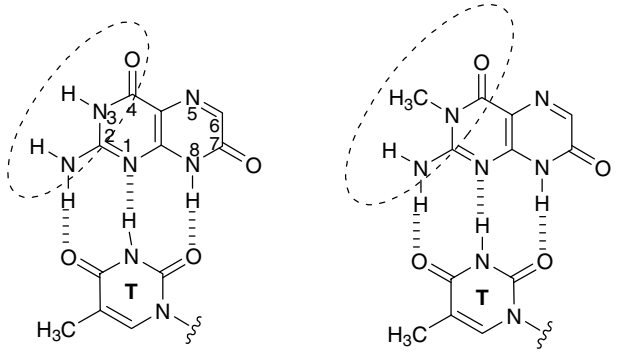
doi:10.1016/j.bmcl.2007.04.033

increased stacking interaction between a ligand, 2,3-dimethyl-pterin, and the flanking bases.[24e]

A recently reported ligand, amiloride,[24d] offers higher binding affinity for T, compared to riboflavin.[24b] Although both amiloride and riboflavin can selectively bind to T with high affinity, these ligands cannot sufficiently differentiate T from C and the difference in the binding constants is within about one order of magnitude. Accordingly, it is highly desired to develop a new ligand which can attain the selective detection of SNPs related to T or C. Several strategies such as introducing substituents into nucleotides and/or ligands have been proposed to improve the binding affinity of ligands and stabilization of DNA duplexes.[25–27] As one such approach, introduction of alkyl groups into the nucleobase and/or ligands in a DNA strand has been known to increase the stacking interaction and the binding ability of ligands with a target nucleobase.[24e,26]

Here we report new fluorescent ligands with a high binding affinity for T via three-point hydrogen bonding interaction, such as **IX** (2-aminopteridine-4,7-(3H,8H)-dione) and **3-MIX** (2-amino-3-methylpteridine-4,7-(3H,8H)-dione) shown in Scheme 1. In Scheme 1, **IX** possesses two sets of hydrogen bond forming sites, one of which is able to bind with C (encircled with the dotted line) and the other is able to bind with T. However, **IX** shows a moderate quenching effect on recognition of both T and C, and the 1:1 binding constants of **IX** with T and C are elucidated as 5.0×10^5, 2.0×10^5 M^{-1}, respectively, at 5 °C. In contrast to **IX**, **3-MIX** has a methyl substituent at the N-3 position of **IX**, which is expected to prevent the binding of the ligand with C and to enhance the selectivity for T against C.

The effect of a methyl group on the binding efficiency of **3-MIX** with an oligo-DNA duplex containing an AP site was first investigated by measurements of melting temperature (T_m). The oligo-DNA duplex used in this experiment is 5′-TCC AGX GCA AC-3′/3′-AGG TCY CGT TG-5′, where X is an AP site composed of a propylene residue, Spacer C3, and Y is G, C, A, or T. As shown in Table 1, **IX** gives an increase in T_m (ΔT_m) of



IX-thymine (T) **3-MIX**-thymine (T)

Scheme 1. Structures of **IX** and **3-MIX** and their possible hydrogen bonding patterns with T.

Table 1. Melting temperature[a] (T_m; °C) of 11-meric ODN duplex containing an AP-site in the absence and presence of **IX** or **3-MIX**

Target base (Y)	Absence of ligand ($T_{m(-)}$)	Presence of **IX** ($T_{m(+)}$) and (ΔT_m)	Presence of **3-MIX** ($T_{m(+)}$) and (ΔT_m)
G	34.0	33.6 (−0.4)	34.9 (+0.9)
C	29.2	33.7 (+4.5)	29.6 (+0.4)
A	33.8	36.0 (+2.2)	35.6 (+1.8)
T	27.9	32.7 (+4.8)	33.9 (+6.0)

[a] ±0.5 °C, each value is the average of at least five measurements. [DNA duplex] = 30.0 μM, [ligand] = 150 μM in a solution buffered to pH 7.0 (10 mM sodium cacodylate) containing 100 mM NaCl and 1.0 mM EDTA. DNA duplex (5′-TCC AGX GCA AC-3′/3′-AGG TCY CGT TG-5′, X = AP site, Y = target bases).

+4.8 °C and 4.5 °C when the nucleobases opposite the AP site are T and C, respectively, in the presence of an oligo-DNA duplex containing an AP site. The stabilization of the oligo-DNA duplex by **IX** is almost identical for both target bases T and C. In contrast, **3-MIX** gives ΔT_m of +6.0 °C and +0.4 °C for the target bases of T and C, respectively. Obviously, **3-MIX** shows higher stability of the oligo-DNA duplex than **IX** when the target base is T, and the stability of the oligo-DNA duplex is not enhanced by **3-MIX** compared with **IX** when the target base is C. This result clearly indicates that introducing a methyl group at the N-3 position of **IX** prevents **3-MIX** from a hydrogen bonding interaction with C and the selectivity for T against C is much improved when **3-MIX** is used as a ligand.

The binding behavior of **IX** and **3-MIX** with the oligo-DNA duplex containing an AP site was further examined by fluorescence measurements. As is shown in Figure 1, **IX** exhibits significant quenching of its fluorescence upon binding with the target nucleobase, and the quenching efficiency is in the order of G < A < C < T in accordance with T_m measurements, while almost no quenching is observed in the presence of normal duplexes containing no AP sites (full-matched DNA). The fluorescence response of **3-MIX** upon binding with the target nucleobase shows stronger quenching than **IX** in the order of C < G < A < T as shown in Figure 2, and it can be easily recognized that highly selective detection of T against C is attained by using **3-MIX** as a ligand. From the fluorescence titration analysis (See Supporting information), as given in Table 2, the 1:1 binding constant between **3-MIX** and T reaches 1.5×10^6 M^{-1}, which is atleast nearly three times larger than that of **IX** and T. Similarly, the 1:1 binding constant between **IX** and C is 0.2×10^6 M^{-1}, at the same time no binding constant could be obtained between **3-MIX** and C, it can be clearly seen that, the introduction of methyl group at N-3 position of **IX** restricted the binding for cytosine with the developed ligand **3-MIX**.

IX and **3-MIX** show T-selective response in fluorescence quenching when both flanking bases of the AP site are G, and the sequence is given by 5′-GXG-3′/3′-CYC-5′, where X and Y are the AP site and the target base, respectively. Next, we examined the effect of the flanking sequences on the binding of **IX** or **3-MIX** to T in the oligo-DNA duplex by fluorescence measurements in order

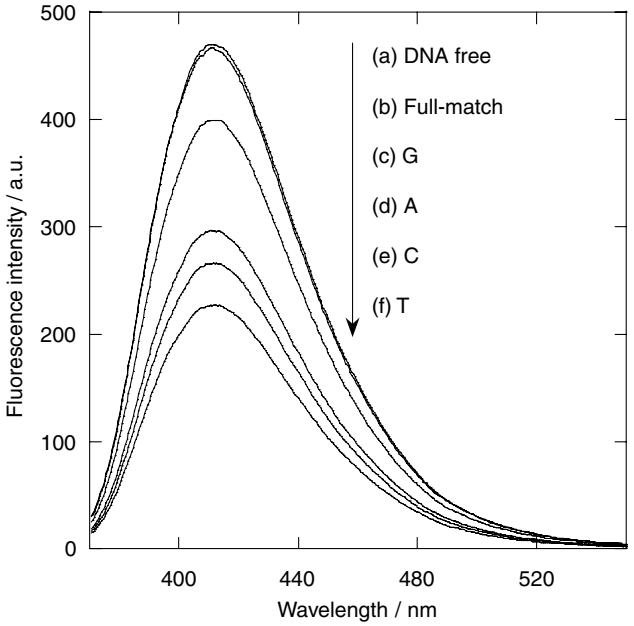


Figure 1. Fluorescence spectra of **IX** in the presence of AP site-containing DNA duplexes (5′-TCC AGX GCA AC-3′/3′-AGG TCY CGT TG-5′, X = AP site, Y = target bases). [DNA duplex] = 10.0 μM, [ligand] = 10.0 μM in a solution buffered to pH 7.0 (10 mM sodium cacodylate) containing 100 mM NaCl and 1.0 mM EDTA. Excitation wavelength, 365 nm. Temperature 5 °C.

to confirm the applicability of these ligands for SNP typing related to T. All possible 16 flanking sequences were examined for the oligo-DNA duplex, 5′-TCT GCG TCC AX′C AAC GCA CAC-3′/3′-AGA CGC AGG TY′G TTG CGT GTG-5′, where X′ is the AP site and

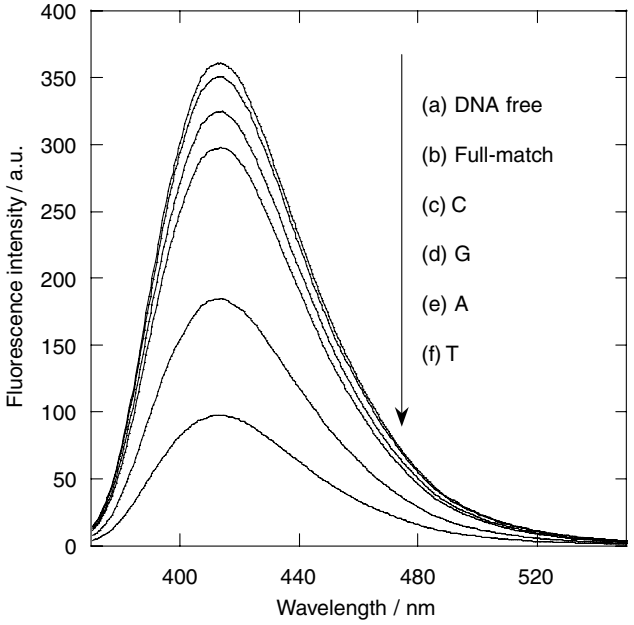


Figure 2. Fluorescence spectra of **3-MIX** in the presence of AP site-containing DNA duplexes (5′-TCC AGX GCA AC-3′/3′-AGG TCY CGT TG-5′, X = AP site, Y = target bases). [DNA duplex] = 10.0 μM, [ligand] = 10.0 μM in a solutions buffered to pH 7.0 (10 mM sodium cacodylate) containing 100 mM NaCl and 1.0 mM EDTA. Excitation wavelength, 365 nm. Temperature 5 °C.

Table 2. Binding constants $K_{1:1}$ at 5 °C for the binding of **IX**[a] and **3-MIX**[b] with each target base. (See Supporting information)

Target base	$K_{1:1}$ of **IX**/10^6 M^{-1}	$K_{1:1}$ of **3-MIX**/10^6 M^{-1}
C	0.2 ± 0.05	Very weak
T	0.6 ± 0.05	1.5 ± 0.04

[a] Concentrations of [**IX**] = 5.0 μM, [DNA duplex] = 1.0–30.0 μM.
[b] [**3-MIX**] = 1.0 μM, and [DNA duplex] = 1.0–6.0 μM, in a solution buffered to pH 7.0 (10 mM sodium cacodylate) containing 100 mM NaCl and 1.0 mM EDTA. DNA duplex (5′-TCC AGX GCA AC-3′/3′-AGG TCY CGT TG-5′, X = AP site; Y = C or T).

its flanking nucleotides and Y′ is the target base T and its flanking nucleotides. The results are shown in Figure 3. In this figure, strong quenching is recognized when the DNA sequence containing flanking nucleotides is CXC/GTG (12), in comparison to the DNA sequence containing GXG/CTC (7), the quenching efficiency is more than two times higher (Supporting information Table S1). This sequence-dependent fluorescence behavior could be ascribed to the difference in the stacking efficiency between the ligand and the various flanking bases.

We next applied the present system for the detection of the T/C mutation present in the cancer repressor gene

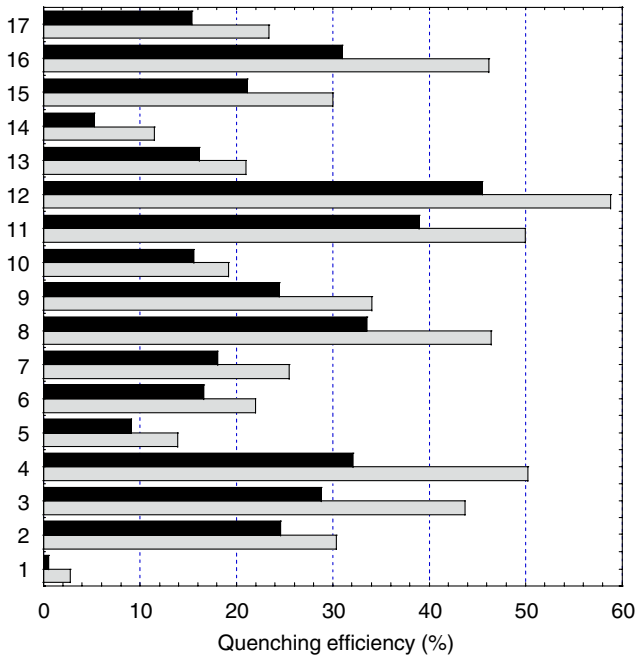


Figure 3. Fluorescence quenching efficiency of **IX** (black bars) and **3-MIX** (gray bars) with different flanking nucleotides (5′-TCT GCG TCC AX′C AAC GCA CAC-3′/3′-AGA CGC AGG TY′G TTG CGT GTG-5′, and X′/Y′ are (1) TTT/AAA, (2) ATA/TXT, (3) ATC/GXT, (4) ATG/CXT, (5) ATT/AXT, (6) CTA/TXG, (7) CTC/GXG, (8) CTG/CXG, (9) CTT/AXG, (10) GTA/TXC, (11) GTC/GXC, (12) GTG/CXC, (13) GTT/AXC, (14) TTA/TXA, (15) TTC/GXA, (16) TTG/CXA, (17) TTT/AXA, X = AP site). Quenching efficiency (%) was calculated by $(I_o - I)/I_o \times 100$, where I_o and I denote fluorescence intensities of **IX** or **3-MIX** in the absence and presence of a DNA duplex, respectively. [DNA duplex] = 1.0 μM, [ligand] = 1.0 μM in a solution buffered to pH 7.0 (10 mM sodium cacodylate) containing 100 mM NaCl and 1.0 mM EDTA. Excitation wavelength, 365 nm.

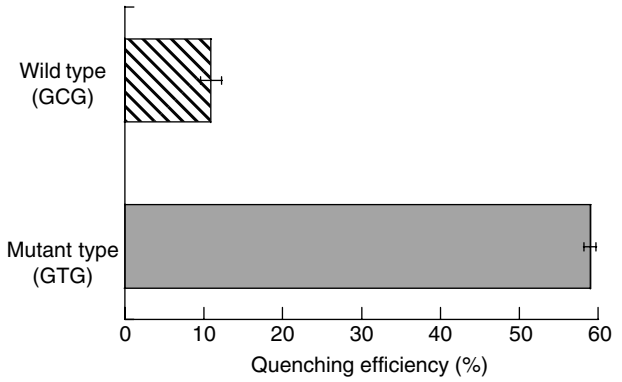


Figure 4. Fluorescence detection of the T/C mutation present in the p53 gene. [DNA duplex] = 1.0 μM; [**3-MIX**] = 1.0 μM; Excitation wavelength 365 nm; Emission 412 nm. Temperature 5 °C. All sample solutions were buffered to pH 7.0 with 10 mM sodium cacodylate containing 100 mM NaCl and 1.0 mM EDTA. DNA duplex (5′-AGC TTT GAG GYG CGT GTT TGT-3′/3′-TCG AAA CTC CXC GCA CAA ACA-5′, X = AP site, Y = C and/or T). Error bars denote the standard deviation of at least three measurements.

p53 of exon 8 at codon 272.[28,29] As is shown in Figure 4, the fluorescence of **3-MIX** is significantly quenched (up to 59%), for the wild type sequence (5′-AGC TTT GAG GTG CGT GTT TGT-3′), while moderate quenching (about 10%) is observed for the mutant sequence (5′-AGC TTT GAG GCG CGT GTT TGT-3′). Therefore, the present method is applicable for the SNP typing related to T.

In summary, we demonstrated that introducing a methyl group into a ligand could tune the base selectivity and enhance the binding affinity of the ligand. Actually, introduction of a methyl group into **IX** resulted in improvement of both binding affinity and selectivity for T against C, which could lead to the efficient detection of the T/C mutation in the p53 gene. The present SNP typing method has advantages of not requiring any special enzymes and labeling fluorophores, compared with conventional methods, although it requires asymmetric PCR method to attain enough discrimination of mutations at present. Further investigation on the detailed binding mechanism and development of more powerful ligands are in progress to attain efficient control of base selectivity and high binding affinity.

Acknowledgments

This work was partially supported by Grants-in-Aid for Scientific Research (A), No. 17205009, for Scientific Research (B), No. 18350039, and for the COE Project, Giant Molecules and Complex Systems, from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.04.033.

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