A Pyrazine-based Fluorescence-enhancing Ligand with a High Selectivity for Thymine in AP Site-containing DNA Duplexes

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A fluorescent pyrazine derivative, 3,5-diamino-6-chloro-2-pyrazine carbonitrile (DCPC), is presented as a promising light-up ligand for single-nucleotide polymorphisms (SNPs) typing. In solutions buffered to pH 7.0 ($I = 0.11$ M, at 5°C), DCPC can bind to thymine selectively over other nucleobases opposite an abasic site in DNA duplexes (5'-GTGTG CGTTG ANA TGGAC GCAGA-3'/3'-CACAC GCAAC TXT ACCTG CGTCT-5', X = abasic site, N = target nucleotide) with a dissociation constant of 2.6 μ M. The binding of DCPC is accompanied by a significant enhancement of its fluorescence (λ_{max} , 412 nm), and the response is highly selective to thymine base. These binding and sensing properties allow a clear detection of thymine-related mutations present in polymerase chain reaction (PCR) amplification products.

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Studies on the chemistry of DNA-binding drugs and/or low-molecular-weight ligands are of on-going interest due to their promising functions and biological activities, including their anti-cancer properties and ability to regulate gene expression.¹⁻⁴ Of particular interest to us is the development of a class of ligands suitable for gene detection, especially for single-nucleotide polymorphisms (SNPs) typing.⁵

We have recently found a series of aromatic ligands that can selectively bind to a nucleobase opposite to an abasic site (AP site) in DNA duplexes,⁶⁻¹⁶ and have proposed a new strategy of ligand-based fluorescence assay for SNPs typing. In contrast to typical DNA-binding ligands capable of targeting double-stranded DNAs by intercalation or groove binding,^{17,18} it is a characteristic of our ligands to target non-Watson-Crick base-pairing sites in DNA duplexes, where the binding is promoted by a pseudo-base pairing along the Watson-Crick edge of intrahelical target nucleobases. Fluorescence signaling is thus obtained by direct interactions between ligands and target nucleobases, and the SNP genotype of samples can be clearly distinguished by combining ligands with selectivity for respective target nucleobases. However, our assay is based on the monitoring of binding-induced fluorescence *quenching* of ligands: as has been observed for fluorescent nucleotide analogs, such as 2-aminopurine,¹⁹ the fluorescence of ligands is significantly quenched due to stacking with nucleobases flanking the AP site. Further efforts are therefore necessary to develop ligands that exhibit fluorescence-enhancement and/or ratiometric emission responses, in order to make an assay more suitable for practical use.

In this work, we successfully discovered a class of light-up ligands upon binding to AP site-containing DNA duplexes. The present ligand, 3,5-diamino-6-chloro-2-pyrazine carbonitrile

(DCPC, Fig. 1), is capable of selectively binding to thymine over other nucleobases, and the binding event is accompanied by a significant enhancement of its fluorescence. While the sensing function depends on nucleobases flanking the AP site, the response of DCPC is almost specific to the thymine base, making it possible to detect thymine-related mutations present in polymerase chain reaction (PCR) amplification products.

Figure 2 shows typical fluorescence responses of DCPC (10 μ M) to 23-mer AP site-containing DNA duplexes (5'-GTGTG CGTTG ANA TGGAC GCAGA-3'/3'-CACAC GCAAC TXT ACCTG CGTCT-5', X = AP site; Spacer-C3, N = target nucleotide), as measured in solutions buffered to pH 7.0 ($I = 0.11$ M, at 5°C). As can be seen from Fig. 2, the response strongly depends on nucleobases opposite the AP site. In the absence of DNAs, the ligand shows a very weak emission with a maximum at 412 nm, and almost no response is observed for guanine, even in the presence of 5 equiv. of DNA (50 μ M). Similarly, the response of DCPC is only moderate for adenine and cytosine. By contrast, DCPC exhibits a significant enhancement of its fluorescence for thymine. In the presence of 1 equiv. of DNA (10 μ M), the emission intensity at 412 nm is indeed enhanced by a factor of 25, and this is accompanied by a 3 nm red-shift of the emission maximum. The response for thymine is concentration-dependent, which is explained by 1:1 binding with a dissociation constant, K_d , of 2.6 μ M (inset of

Fig. 1 Chemical structures of pyrazines examined in this work.

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Fig. 2 Fluorescence responses of DCPC (10 μ M) to AP site-containing DNA duplexes (10 μ M; 5'-GTGTG CGTTG ANA TGGAC GCAGA-3'/3'-CACAC GCAAC TXT ACCTG CGTCT-5', X = AP site; Spacer-C3, N = target nucleotide) in solutions buffered to pH 7.0 (10 mM sodium cacodylate) containing 100 mM NaCl and 1.0 mM EDTA. Excitation wavelength, 371.5 nm. Temperature, 5°C. Inset: Nonlinear regression analysis of changes in the fluorescence intensity ratio at 412 nm based on a 1:1 binding isotherm model. F and F_0 denote the fluorescence intensities of DCPC in the presence and absence of DNA duplexes, respectively.

Fig. 2). Apparently, DCPC does work as a light-up ligand with a useful affinity and selectivity for thymine in AP site-containing DNA duplexes.

An examination of the effect of flanking nucleobases on fluorescence responses reveals that DCPC is able to exhibit the emission enhancement to the sequences containing no guanines adjacent to the AP site. As summarized in Table 1, DCPC covers 9 sequences among a total of 16 sequences, and, in particular, the response is significant and effective when the AP site is flanked by adenine or thymine.

Interestingly, structurally related amiloride (Fig. 1),¹⁰ a pyrazine derivative having a positively charged guanidinium group at the 2-position of the ring, is found to also show emission enhancement upon binding to thymine. Under identical conditions given in Fig. 2 (pH 7.0, $I = 0.11$ M, at 5°C), amiloride shows a stronger binding affinity for thymine ($K_d = 0.25$ μ M), which is indeed an advantage over DCPC. However, as compared to DCPC, the light-up response of amiloride (λ_{max} : 415 nm) is only moderate ($F/F_0 = 4.3$), and is obtained only for 4 sequences, as summarized in Table 1. It is therefore highly likely that an electron-withdrawing CN group is responsible for the better response functions of DCPC, by regulating the electronic properties of the pyrazine ring.

Finally, DCPC was applied to an analysis of thymine-related mutation present in 141-mer DNAs (*p53* gene,²⁰ codon 280 G > T). After asymmetric PCR amplification,^{21,22} the products were analyzed at 5°C in a buffer solution (pH 7.0, 100 mM sodium cacodylate) containing 1.6 mM EDTA, 1.0 μ M DCPC and 5.0 μ M AP site-containing 21-mer probe DNA. A fluorescence enhancement of DCPC (excitation wavelength: 380 nm, analysis: 412 nm) is clearly observed for a thymine-containing mutant-type sequence (ATA: 530%), while the response is almost negligible for a wild-type sequence (AGA: -5%). The analysis requires no time-consuming steps, such as the purification of PCR products, and careful control of the temperature, and the result is readily obtained after PCR.

In summary, we have demonstrated a pyrazine-based light-up ligand applicable to the detection of thymine-related mutations. Further studies are in progress to obtain more details about

Table 1 Effect of flanking nucleotides on the fluorescence responses (F/F_0) of ligands to thymines in 23-mer DNA duplexes^a

5'-zTz'-3'/3'-wXw-5'	DCPC ^b	Amiloride ^c
5'-TTC-3'/3'-AXG-5'	0.56	0.34
5'-GTC-3'/3'-CXG-5'	0.53	0.30
5'-CTC-3'/3'-GXG-5'	0.59	0.29
5'-ATC-3'/3'-TXG-5'	0.53	0.37
5'-TTT-3'/3'-AXA-5'	22	1.7
5'-GTT-3'/3'-CXA-5'	11	0.71
5'-CTT-3'/3'-GXA-5'	0.66	0.22
5'-ATT-3'/3'-TXA-5'	39	2.2
5'-TTG-3'/3'-AXC-5'	2.6	0.27
5'-GTG-3'/3'-CXC-5'	1.8	0.22
5'-CTG-3'/3'-GXC-5'	0.51	0.20
5'-ATG-3'/3'-TXC-5'	2.5	0.36
5'-TTA-3'/3'-AXT-5'	19	1.7
5'-GTA-3'/3'-CXT-5'	6.9	0.49
5'-CTA-3'/3'-GXT-5'	0.64	0.30
5'-ATA-3'/3'-TXT-5'	27	3.0

a. Measured in solutions buffered to pH 7.0 with 10 mM sodium cacodylate, containing 100 mM NaCl and 1.0 mM EDTA. Temperature, 5°C. F and F_0 denote the fluorescence intensities of ligands in the presence and absence of DNA duplexes, respectively. DNA duplex: 5'-GTGTG CGTTG zTz' TGGAC GCAGA-3'/3'-CACAC GCAAC wXw' ACCTG CGTCT-5', X = AP site; Spacer-C3.

b. Excitation, 371.5 nm; detection, 412 nm. [Ligand] = 15 μ M; [DNA duplex] = 15 μ M.

c. Excitation, 380.5 nm; detection, 415 nm. [Ligand] = 1.0 μ M; [DNA duplex] = 1.0 μ M.

pyrazines-DNA interactions for the development of this class of ligands suitable for gene detection.

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 22. Asymmetric PCR²¹ amplification of 141-mer sense strands of *p53* gene²⁰ was done with a 24-mer forward primer (5'-GTAGTGGTAATCTACTGGGACGGA-3') and a 23-mer reverse primer (5'-CTCGCTTAGTGCTCCCTGGGGGC-3'). The reaction solution (100 µl) contained dNTPs (2.5 mM each, 8 µl), 10 × PCR buffer (10 µl; TaKaRa), forward primer (300 pmol), reverse primer (20 pmol), template (0.2 pmol), and Ex-Taq (2.5 U; TaKaRa). PCR conditions: 94°C for 5 min, followed by 50 cycles of 96°C for 30 s, 57°C for 30 s, and 72°C for 30 s, and then 72°C for 5 min and kept at 4°C. 141-mer PCR product: 5'-GT AGT GGT AAT CTA CTG GGA CGG AAC AGC TTT GAG GTG CGT GTT TGT GCC TGT CCT GGG AYA GAC CGG CGC ACA GAG GAA GAG AAT CTC CGC AAG AAA GGG GAG CCT CAC CAC GAG CTG CCC CCA GGG AGC ACT AAG CGA G-3' (wild type, Y = G; mutant type, Y = T); 21-mer probe DNA: 5'-GCG CCG GTC TXT CCC AGG ACA-3' (X = AP site; Spacer-C3).
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