6,7-Dimethyllumazine as a potential ligand for selective recognition of adenine opposite an abasic site in DNA duplexes†

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6,7-Dimethyllumazine more selectively binds to adenine (A) base opposite the abasic site in DNA duplexes (5′-TCC AGX GCA AC-3′/3′-AGG TCN CGT TG-5′, X = AP site (Spacer C3), N = A, T, C and G) than the other three nucleobases with a dissociation constant K_d of *ca.* 1.0 μM; substituted methyl groups enhance the binding affinity to A and the selectivity for A over T, compared to the parent molecule, lumazine.

Single nucleotide polymorphisms (SNPs) are extremely important as a genetic marker for the identification of disease genes and detection of genetic mutations.[1,2] Thus, simple and quick detection of SNPs is a key issue in today's chemical biology, biotechnology, medicine and pharmacogenomics.[3] Consequently, considerable research efforts have been made to develop high-throughput analysis of SNPs based on short oligo-nucleotides labelled by fluorescent molecules.[4] Nakatani *et al.*[5] have recently synthesized dimeric naphthyridine derivatives that specifically bind to the guanine–guanine,[5a] cytosine–cytosine,[5b] and guanine–adenine[5c] mismatches, and they succeeded in developing a non-gel-based scanning method to detect the ligand-bound SNPs,[5a] using a surface plasmon resonance (SPR) assay. On the other hand, we have also proposed a new strategy of ligand-based fluorescence assay for SNP typing (Fig. 1, left), and have discovered a series of small fluorescence ligands that can bind to a nucleobase opposite an abasic site (AP site) in DNA duplexes.[6–9] In contrast to typical DNA–drug binding ligands (groove binders or intercalators), the AP site in DNA duplexes provides a unique hydrophobic binding pocket which allows the pseudo-base pairing of ligands with intrahelical nucleobases along the Watson–Crick edge,[10] where the ligand is stacked with two nucleobases flanking the AP site. In combination with AP site-containing DNA duplexes, we have reported a series of ligands such as 2-amino-7-methyl-1,8-naphthyridine (AMND),[6] 2-amino-6,7-dimethyl-4-hydroxypteridine,[7] amiloride,[8] and alloxazine[9] with high affinities for cytosine (C), guanine (G), thymine (T) and adenine (A), respectively. Among these base-selective ligands, developing an efficient A-selective ligand still remains as a task to be pursued, since alloxazine cannot sufficiently discriminate A (the 1 : 1 binding constant, $K_{11} = 1.21 \times 10^6$ M^{-1}) from T ($K_{11} = 0.72 \times 10^6$ M^{-1}).[9] Accordingly, it is highly desired to develop a new ligand to attain the selective detection of SNPs related to adenine. 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.[7b,11] 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[11] and the binding ability of ligands with a target nucleobase.[7b]

Here we report on new fluorescent ligands with a high binding affinity for adenine, such as 6,7-dimethyllumazine. Taking into account the structure of previously reported ligands as a clue for binding to adenine, we have found that lumazine and its derivative, 6,7-dimethyllumazine, possess suitable hydrogen-bonding sites with high complementarity for adenine (Fig. 1, middle), while fluorescence properties of lumazine and its derivatives have been examined upon incorporation into DNA.[12] Introduction of methyl groups to lumazine at the 6,7-positions is a key idea to improve the selectivity for adenine base; it is highly likely that introducing an electron donating group neighboring the hetero atoms (N-8 position of lumazine) will enhance the electron density at the binding site of 6,7-dimethyllumazine to adenine base. Therefore, the high selectivity in complexation, with accompanying significant fluorescence quenching, is obtained exclusively with the adenine nucleobase in aqueous media.

First, we used fluorescence measurements to examine the binding behavior of lumazine and 6,7-dimethyllumazine with each target base opposite the AP site in DNA duplexes (5′-TCCA GXG CAAC-3′/3′-AGGT CNC GTTG-5′,

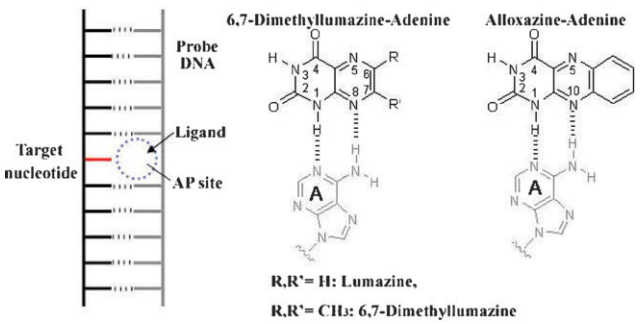

Fig. 1 Schematic illustration of the ligand-based fluorescence detection of single-nucleotide polymorphisms in combination with an AP site-containing probe DNA (left). Possible binding modes for adenine by lumazine or 6,7-dimethyllumazine (middle) and alloxazine (right).

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† Electronic supplementary information (ESI) available: Synthesis of 6,7-dimethyllumazine (Scheme S1), absorption and fluorescence data (Fig. S1–S5), salt dependence (Fig. S6 and Table S1), ITC data (Fig. S7 and S8), DNA sequence effect on fluorescence (Fig. S9). See DOI: 10.1039/b816876h

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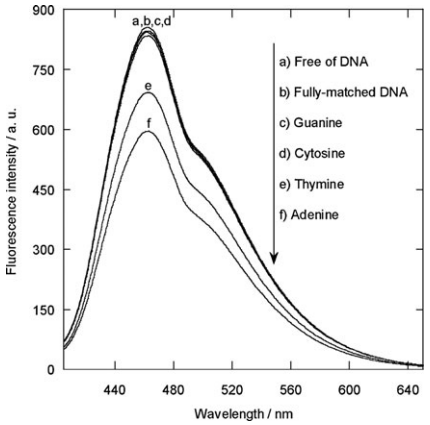

Fig. 2 Fluorescence spectra of lumazine in the absence and presence of AP site-containing DNA duplexes (5′-TCCA G**X**G CAAC-3′/3′-AGGT C**N**C GTTG-5′, **X** = AP site, **N** = G, C, A, T). [DNA duplex] = 10.0 μM, [lumazine] = 10.0 μM in a solution buffered to pH 7.0 (10 mM sodium cacodylate containing 100 mM NaCl and 1.0 mM EDTA). Excitation wavelength = 335 nm, 5 °C.

X = Spacer C3, **N** = G, C, A, T) by fluorescence measurements. As is shown in Fig. 2, lumazine exhibits significant quenching of its fluorescence upon binding with the target adenine base, and the quenching efficiency is in the order of G < C < T < A, while almost no quenching is observed in the presence of normal duplexes (fully matched DNA) containing no AP sites. The fluorescence response of 6,7-dimethyllumazine upon binding with the target nucleobase shows higher quenching than lumazine in the order of G < C < T << A as shown in Fig. 3, and it can be seen that highly selective detection of A over T, C and G is attained by using 6,7-dimethyllumazine as a ligand. From the fluorescence titration analysis (ESI, Fig. S2†), K_{11} for lumazine with adenine is 0.085×10^6 M^{-1}, and for 6,7-dimethyllumazine with adenine, it is 0.83×10^6 M^{-1} (see ESI Fig. S3†) which is about one order

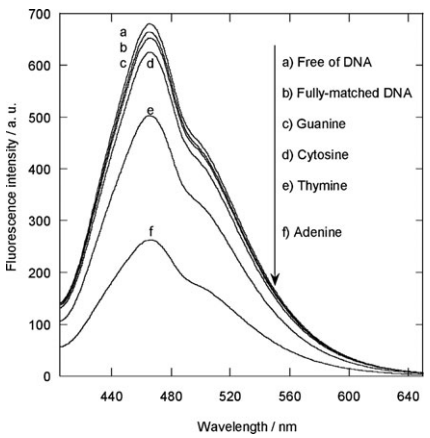

Fig. 3 Fluorescence spectra of 6,7-dimethyllumazine in the absence and presence of AP site-containing DNA duplexes (5′-TCCA G**X**G CAAC-3′/3′-AGGT C**N**C GTTG-5′, **X** = AP site, **N** = G, C, A, T). [DNA duplex] = 5.0 μM, [6,7-dimethyllumazine] = 5.0 μM in a solution buffered to pH 7.0 (10 mM sodium cacodylate containing 100 mM NaCl and 1.0 mM EDTA). Excitation wavelength = 350.5 nm, 5 °C.

magnitude higher than that of lumazine with adenine. Similarly, the binding constants of lumazine and 6,7-dimethyllumazine with thymine are 0.03×10^6 M^{-1} and 0.07×10^6 M^{-1}, respectively. As expected, it is clear that the difference in the binding constants of adenine against thymine is achieved with a large discrimination ratio using 6,7-dimethyllumazine. Interestingly, the binding affinity of 6,7-dimethyllumazine is further strengthened by the use of 23-mer duplex (5′-TCTGC GTCCA G**X**G CAACG CACAC-3′/3′-AGACG CAGGT CA**C** GTTGC GTGTG-5′, **X** = Spacer C3, ESI, Fig. S4†) containing adenine opposite the AP site (K_{11} = 1.0×10^6 M^{-1}). In contrast with lumazine and the previously reported ligand, alloxazine[9] (ESI Fig. S5†), 6,7-dimethyllumazine has a striking ability to recognize A over T, C and G.

As shown in Fig. 3, the binding affinity and the selectivity of 6,7-dimethyllumazine for adenine exhibit significant improvement in comparison to lumazine (Fig. 2) due to the increased stacking interactions between 6,7-dimethyllumazine and the bases flanking the AP site by introducing two methyl groups to lumazine. In the case of alloxazine and its derivative (lumichrome: 7,8-dimethylalloxazine),[9] the base-selectivity was changed from adenine to thymine by adding methyl groups at 7 and 8 positions of alloxazine. On the other hand, the base-selectivity is kept for adenine and the binding affinity is enhanced for 6,7-dimethyllumazine compared to lumazine. The enhancement in the binding affinity can be ascribed to the increased hydrophobicity and polarizability of methyl groups on the ligand. In addition, the presence of two methyl groups (*i.e.*, electron-donating groups) neighboring the binding site, the electron density at the N-8 position of the lumazine ring significantly increases, hence 6,7-dimethyllumazine prefers adenine-recognition (at N-1 and N-8 positions) more strongly than lumazine.

Further, the binding constants of lumazine and 6,7-dimethyllumazine were also determined at different salt concentrations using fluorescence measurements. As is shown in ESI Fig. S6,† the binding constants for lumazine and 6,7-dimethyllumazine decrease slightly as the salt concentration increases. These results indicate that the binding of lumazine or 6,7-dimethyllumazine to DNA is accompanied by rather less release of counterions. From the double logarithmic linear fit to binding constants under different salt concentration, a slope of −0.061 was estimated, by which an apparent charge of 6,7-dimethyllumazine was obtained (Z = +0.07) for the binding to adenine in AP site-containing DNA duplexes according to M. T. Record *et al.*[13] From the dependence of the binding constant on salt concentration, the binding free energies of polyelectrolyte contribution (ΔG_{pe}) and non-polyelectrolyte contribution (ΔG_t) were calculated based on the polyelectrolyte theory by Record *et al.*[13] The polyelectrolyte contribution to the binding free energy change (ΔG_{obs}) is small for the 6,7-dimethyllumazine binding (ΔG_{pe} = −0.074 kcal mol^{-1}) and the lumazine binding (ΔG_{pe} = −0.19 kcal mol^{-1}) as listed in Table 1. Whereas, ΔG_t of the 6,7-dimethyllumazine binding and the lumazine binding are −7.46 kcal mol^{-1} and −6.08 kcal mol^{-1}, respectively. The values of ΔG_t reveal that the non-polyelectrolyte component of binding is fundamental for the stabilization of the complex between lumazine or 6,7-dimethyllumazine complex and DNA. Moreover, it also indicates that the increase in the binding

Table 1 Thermodynamic parameters for the binding of lumazine or 6,7-dimethyllumazine to adenine in the 11-mer AP site-containing DNA duplex[a]

	K_{obs}/M^{-1}	ΔG_{obs}/ kcal mol^{-1}	$-SK$	ΔG_{pe}/ kcal mol^{-1}	ΔG_t/ kcal mol^{-1}	ΔH_{obs}/ kcal mol^{-1}	$T\Delta S_{obs}$/ kcal mol^{-1}
Lumazine	0.085 (±0.002) × 10^6	−6.27 (±0.01)	0.153 (±0.012)	−0.19 (±0.01)	−6.08 (±0.02)	−9.5 (±0.26)	−3.23 (±0.27)
6,7-Dimethyllumazine	0.83 (±0.02) × 10^6	−7.53 (±0.01)	0.061 (±0.0047)	−0.074 (±0.006)	−7.46 (±0.02)	−14.0 (±0.08)	−6.47 (±0.09)

[a] K_{obs} (M^{-1}), determined by fluorescence titration experiments, is the 1 : 1 binding constant in 110 mM Na^+ at 5 °C ([sodium cacodylate] = 10 mM, [EDTA] = 1 mM, [NaCl] = 100 mM, pH 7.0). ΔG_{obs} is the observed binding free energy calculated from $\Delta G_{obs} = -RT\ln K_{obs}$. SK is the slope of the plot of log K_{obs} *versus* log a_{Na^+}. ΔG_{pe} and ΔG_t are the polyelectrolyte and non-polyelectrolyte contributions to the binding free energy ($\Delta G_{pe} = (-SK)RT\ln[Na^+]$) evaluated at 110 mM Na^+. ΔH_{obs} was determined by ITC at 5 °C. $T\Delta S_{obs}$ was calculated from $T\Delta S_{obs} = \Delta H_{obs} - \Delta G_{obs}$. DNA duplex: 5′-TCC AG**X** GCA AC-3′/3′-AGG TC**A** CGT TG-5′, **X** = AP site; **A** = adenine. Errors: K_{obs}; the SD by three independent repeated measurements, ΔH_{obs}; fitting error.

constant for 6,7-dimethyllumazine is mainly due to a large non-polyelectrolyte contribution.

For analyzing the energetics and stoichiometry of the interaction, the binding of 6,7-dimethyllumazine with adenine was further characterized by isothermal titration calorimetry (ITC) experiments. As shown in ESI Fig. S8,† the addition of the duplex aliquots (200 μM) into the solution containing 6,7-dimethyllumazine (20 μM) caused a large exothermic heat of reaction, and the corrected binding isotherm was obtained after the heat of dilution was subtracted. The resulting titration curve was able to be best fitted using a model that assumes a single set of identical binding sites with a stoichiometry n of 1.1, suggesting a 1 : 1 binding. The thermodynamic parameters of the interactions for the 6,7-dimethyllumazine-adenine and lumazine-adenine are summarized in Table 1. As can be seen there, the ligand-duplex interaction is enthalpy-driven for both lumazine and 6,7-dimethyllumazine. Compared to the enthalpy loss of lumazine ($\Delta H_{obs} = -9.5$ kcal mol^{-1}), the increase in enthalpy loss of 6,7-dimethyllumazine ($\Delta H_{obs} = -14.0$ kcal mol^{-1}) can be ascribed primarily to the effect of the introduced methyl groups. Despite the value of entropy loss from lumazine to 6,7-dimethyllumazine being negative ($\Delta(T\Delta S) = -3.24$ kcal mol^{-1}), it is highly compensated by the less negative value of enthalpy ($\Delta\Delta H_{obs} = -4.5$ kcal mol^{-1}), that results in a more favorable free energy change ($\Delta\Delta G_{obs} = -1.26$ kcal mol^{-1}). Therefore the DNA binding constant of 6,7-dimethyllumazine is 11-fold higher than that of lumazine. It is highly likely that the formation of hydrogen bonds and stacking interactions are more effective in the 6,7-dimethyllumazine-duplex binding.

In summary, a new class of site-recognition ligand, 6,7-dimethyllumazine, has been developed for fluorescence detection of the adenine base by using the AP site as the field of molecular recognition. 6,7-Dimethyllumazine was able to selectively recognize adenine base with 1 : 1 binding affinity of 1.0×10^6 M^{-1}, accompanied by significant fluorescence quenching. It was demonstrated that the hydrogen bonding and the stacking interactions with flanking nucleotides played an important role in the complexation of 6,7-dimethyllumazine with adenine at the AP site, which indeed controlled the binding stability and selectivity. While further efforts will be necessary to improve the dependence of fluorescence responses on nucleobases flanking the AP site (ESI Fig. S9†), the new ligand (6,7-dimethyllumazine) would be useful for the detection of adenine-related SNPs, and the results presented here will provide a rational basis for further studies on the design and synthesis of new adenine-selective fluorescent ligands with the improved binding and sensing properties.

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