Strong and selective binding of amiloride to thymine base opposite AP sites in DNA duplexes: simultaneous binding to DNA phosphate backbone†

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Amiloride (*N*-amidino-3,5-diamino-6-chloro-pyrazinecarboxamide hydrochloride) has two sets of hydrogen-bond forming sites suitable for target nucleotides and the phosphodiester DNA backbone by which a thymine base opposite an abasic site in DNA duplexes can be recognized with high selectivity and affinity, and it is applicable to the fluorescence detection of thymidine-related SNPs (single-nucleotide polymorphisms) of PCR amplification products.

Studies on the chemistry of DNA-binding drugs and/or low molecular weight ligands are of ongoing interest due to their promising functions and biological activities, including anti-cancer properties and their abilities to modulate gene expression and fluorescence staining of nucleic acids.[1] Of particular interest to us is the use of small ligands to develop new methods for the detection of SNPs (single-nucleotide polymorphisms).[2,3]

We have recently discovered a series of small aromatic ligands that can bind to a nucleobase opposite an abasic site (AP site) in DNA duplexes, and have proposed a new strategy of ligand-based fluorescence assay for SNPs typing (Fig. 1A).[3] In contrast to typical DNA–drug bindings such as groove binding or intercalation,[4] the AP site in DNA duplexes provides a unique binding pocket which allows the pseudo-base pairing of ligands with intrahelical nucleobases along the Watson–Crick edge, where the ligand is stacked with two nucleobases flanking the AP site. We obtained high selectivity toward target nucleobases with the binding affinity up to the micromolar range in AMND (2-amino-7-methyl-1,8-naphthyridine)–cytosine,[3a,3c] pterin–guanine,[3b] and vitamin B_2–thymine[3d] bindings. However, the binding mode of these AP site-binding ligands is relatively simple, and a more rational design of this class of ligands would be critical for our system. This would also offer a valuable insight into the molecular basis of interactions for the further development of various kinds of DNA-binding drugs.

Here we report on a new class of AP site-binding fluorescence ligands, amiloride (*N*-amidino-3,5-diamino-6-chloro-pyrazinecarboxamide hydrochloride),[5] which has two sets of hydrogen-bond forming sites on either end of the system, *i.e.*, a hydrogen-bonding profile fully complementary to thymine, and a guanidinium moiety suitable for recognition of the phosphodiester DNA backbone (Fig. 1B). Molecular modeling studies indicate that the formation of such a multiple hydrogen-bonded complex is feasible (Fig. 1C). In this work, we investigate the binding of amiloride to 11-mer AP site-containing duplexes (5′-TCC AG<u>X</u> GCA AC-3′/3′-AGG TC<u>Y</u> CGT TG-5′, <u>X</u> = AP site, <u>Y</u> = target), with an emphasis on the effect of the guanidinium moiety, and we demonstrate a highly selective interaction to T with a nanomolar range of binding affinity. We also make a case for the potential use of amiloride for the analysis of T-related SNPs of PCR amplification products.

The binding of amiloride to AP site-containing duplexes was examined firstly by fluorescence measurements. As is shown in Fig. 2A, amiloride exhibits significant quenching of its fluorescence upon addition of DNA duplexes containing T opposite the AP site, while almost no responses are observed in the presence of normal duplexes containing no AP sites (not shown). The resulting titration curve can be analyzed by a 1 : 1 binding isotherm (Fig. 2B), giving a dissociation constant K_d of 150 nM. This value is indeed stronger than the binding affinity of vitamin B_2, a T-selective ligand as reported in our previous work (K_d = 560 nM for T).[3d] Furthermore, as revealed by fluorescence binding titration (Fig. 2B), amiloride is capable of selectively binding to T over C, A and G. The binding affinity for T is one order of magnitude higher than that for the other three nucleotides (K_d/nM: C: 1000; A: 5600; G: 6700). Thus, amiloride has a striking ability to recognize T in AP site-containing DNA duplexes.

For the existence of such a nanomolar range of binding affinity, the positively charged guanidinium moiety is essential. This is clearly supported by a much lower affinity of 3,5-diamino-6-chloro-2-pyrazinecarbonitrile (DCPC), a control ligand that lacks the corresponding monocationic binding site (K_d = 3500 nM for T). Based on the polyelectrolyte theory by Record *et al.*,[6] our examination of salt dependence of binding constants (Supporting Information Fig. S1 and Table S1†) shows that the apparent charge, Z, on DCPC is +0.29 when binding to T in AP site-containing duplexes. In the case of amiloride with a monovalent charge, Z is found to be +1.5, indicating that the binding is accompanied by a distinct release of condensed counter ions that provides an entropically favorable contribution to the binding free energy. At the 110 mM Na^+ concentration, such a polyelectrolyte contribution ΔG_{pe} is calculated as -1.7 kcal mol^{-1}, revealing a significant contribution of ΔG_{pe} to the overall free energy of amiloride–duplex interaction (-8.7 kcal mol^{-1}).

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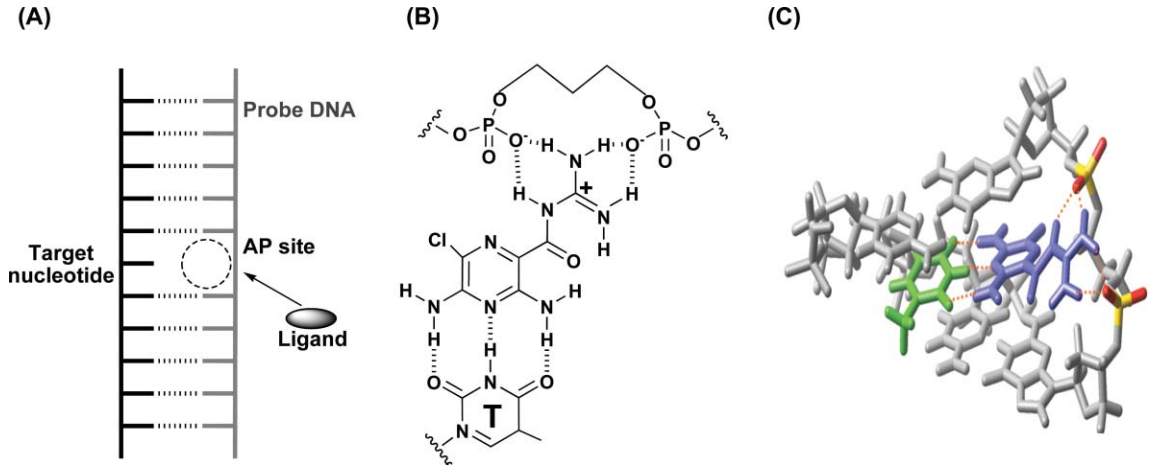

Fig. 1 (A) Schematic illustration of the ligand-based fluorescence detection of single-nucleotide polymorphisms, in combination with an AP site-containing probe DNA. (B) Possible binding mode of amiloride with T in the AP site-containing DNA duplexes. (C) Energy-minimized structure, obtained using MacroModel Ver. 8.5, for the complex between amiloride and DNA duplex (5′-TCC AGX GCA AC-3′/3′-AGG TCT CGT TG-5′, X = AP site, T = target thymine). The amiloride and the thymine base opposite the AP site are colored blue and green, respectively. Oxygen atoms of the phosphate groups flanking the AP site are colored red.

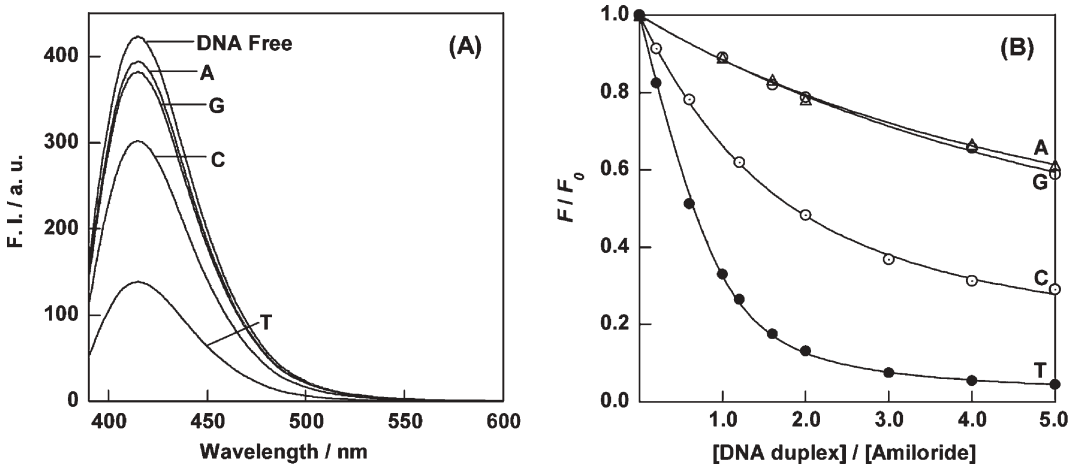

Fig. 2 (A) Fluorescence spectra of amiloride (1.0 μM) in the presence of AP site-containing DNA duplexes (5′-TCC AGX GCA AC-3′/3′-AGG TCY CGT TG-5′, X = AP site, Y = target bases, 1.0 μM) in solutions buffered to pH 7.0 (10 mM sodium cacodylate) containing 100 mM NaCl and 1.0 mM EDTA. Excitation wavelength, 380.5 nm. Temperature, 5 °C. (B) Nonlinear regression analysis of the changes in the fluorescence intensity ratio at 415 nm based on a 1 : 1 binding isotherm model. F and F_0 denote the fluorescence intensities of amiloride in the presence and absence of DNA duplexes, respectively.

The binding of amiloride with T is further characterized by ITC (isothermal titration calorimetry) experiments. The addition of the duplex aliquots into the solution containing amiloride causes a large exothermic heat of reaction while the heat of dilution for the free duplex is only moderate (Supporting Information Fig. S2†). From the analysis of the resulting titration curve, an association constant K_a is obtained as 4.0×10^6 M^{-1} with a stoichiometry n of 1.2 (Table 1). The ligand–nucleotide interaction is enthalpy driven, with ΔG° of -8.6 kcal mol^{-1}, ΔH° of -17.3 kcal mol^{-1} and $T\Delta S^\circ$ of -8.7 kcal mol^{-1} at 10 °C. The comparison with the thermodynamic profile of DCPC ($K_a = 2.3 \times 10^5$ M^{-1}, $n = 1.2$, $\Delta G^\circ = -7.0$ kcal mol^{-1}, $\Delta H^\circ = -15.8$ kcal mol^{-1}, and $T\Delta S^\circ = -8.9$ kcal mol^{-1} at 10 °C) shows that the stronger affinity of amiloride can be ascribed primarily to the favorable gain in enthalpy ($\Delta\Delta H^\circ = -1.4$ kcal mol^{-1}), without further loss of binding entropy ($\Delta(T\Delta S^\circ) = +0.2$ kcal mol^{-1}). It is therefore highly likely that the formation of hydrogen bonds and stacking is more

Table 1 Comparison of the thermodynamic parameters for the DNA binding of amiloride and DCPC[a]

Thermodynamic Parameters	Amiloride	DCPC
n	1.2	1.2
K_a/M^{-1}	4.0×10^6	0.23×10^6
ΔG/kcal mol^{-1}	−8.6	−7.0
ΔH/kcal mol^{-1}	−17.3	−15.8
$T\Delta S$/kcal mol^{-1}	−8.7	−8.9

[a] The parameters were obtained by ITC experiments in solutions buffered to pH 7.0 at 10 °C (Fig. S2). DNA duplex: 5′-TCC AGX GCA AC-3′/3′-AGG TCT CGT TG-5′ (X = AP site, T = target thymine).

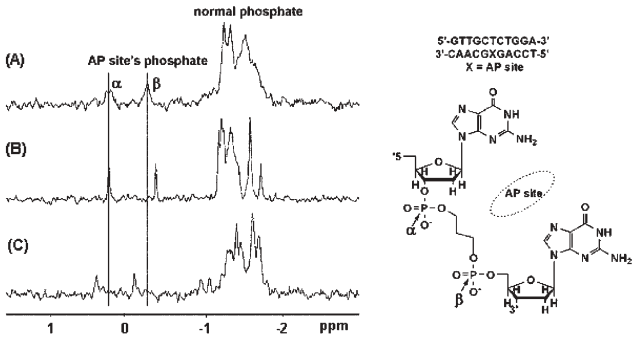

Fig. 3 ^{31}P NMR spectra of AP site-containing DNA duplex (5′-TCC AGX GCA AC-3′/3′-AGG TCT CGT TG-5′, X = AP site, 150 μM) in solutions buffered to pH 7.0 (10 mM sodium cacodylate) containing 100 mM NaCl and 1.0 mM EDTA. (A) DNA alone, (B) DNA–DCPC, and (C) DNA–amiloride. [Ligand], 200 μM. 10% D_2O was added to lock signals. Temperature, 10 °C.

effective in the amiloride–duplex binding, in which the amiloride is trapped inside the AP site through the formation of multiple hydrogen bonds in a bidentate fashion.

^{31}P NMR measurements indicate the occurrence of the hydrogen-bonding interaction between the guanidinium moiety and the DNA phosphate backbone. As is shown in Fig. 3A, the phosphate groups flanking the AP site give two signals at 0.145 ppm (α) and −0.296 ppm (β) which are farther downfield compared to signals from normal phosphate groups; this is a characteristic feature of AP site-containing DNA duplexes.[7] Upon binding with amiloride (Fig. 3C), downfield shifts for both α (0.09 ppm) and β (0.12 ppm) are clearly observed, but such changes in chemical shifts of the AP site's phosphate groups are not observed even in the presence of DCPC (Fig. 3B, upfield shift: α: 0.02 ppm; β: 0.09 ppm). Apparently, the guanidinium moiety of amiloride is interacting with both phosphate groups flanking the AP site, and the binding at the AP site is likely to cause the reorganization of the whole structure of DNA duplexes, as revealed by the changes in the signals from normal phosphate groups.

Finally, preliminary experiments reveal that amiloride is applicable to the analysis of T-related mutation of PCR amplification products, where a significant fluorescence quenching is only observed for the T-containing sequence (Supporting Information Fig. S3†). The analysis requires no time-consuming steps such as purification of the PCR products and careful control of the temperature, and the result is readily obtained after the PCR.

In summary, we have successfully discovered a new class of AP site-binding ligands, amiloride, having a highly selective and strong binding affinity for thymine. The sophisticated combination of hydrogen-bond forming sites for target nucleotides and the phosphodiester DNA backbone is a noteworthy approach to design this class of ligands, and the results presented here will provide a rational basis for the development of SNP detection chemistry based on DNA-binding small molecules.

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