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COMMUNICATION

Highly selective binding of naphthyridine with a trifluoromethyl group to cytosine opposite an abasic site in DNA duplexes†

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We report on highly selective binding of a naphthyridine derivative with a trifluoromethyl group to cytosine opposite an abasic site in DNA duplexes; the binding-induced fluorescence quenching is applicable to the analysis of a C-related single-base mutation in DNAs amplified by PCR.

Small ligands that can bind to DNA duplexes have been developed because of their promise in various applications due to their functionalities such as the regulation of gene expression.[1] These ligands have also been utilized as stain agents for DNA duplexes,[2] with ethidium bromide and Hoechst 33258 being typical examples. Among these ligands, particular efforts have been recently devoted to develop site-selective binding ligands that recognize the intrahelical nucleobases in DNA duplexes,[3] where hydrogen bonding-mediated recognition of target nucleobases is a useful molecular basis for the design of this class of ligand.[4] Indeed, many hydrogen-bonding ligands have been developed for detecting nucleobases at the abasic site (apyrimidinic or apurinic; AP),[5] bulge[4a,c,6] and mismatched sites[7] in DNA duplexes. It has been demonstrated that these ligands are applicable to gene diagnostics and therapy,[4–7] as well as DNA-based biotechnology such as molecular beacons,[8a] aptamers,[8b–d] and nanotechnology.[8e]

2-Amino-1,8-naphthyridine derivatives are useful scaffolds for selective binding to the pyrimidine nucleobases in DNA duplexes. We reported that 2-amino-7-methyl-1,8-naphthyridine (AMND, Fig. 1)[5a,b] showed selective binding to cytosine (C) opposite an AP site in DNA duplexes, and the binding-induced fluorescence quenching was applicable to the analysis of the C-related single base mutation. The additional substitution of methyl groups to AMND enables a significant increase in the binding affinity of the ligand to C.[5c] Similarly, 2,7-diamino-1,8-naphthyridine can bind selectively to the C bulge in DNA duplexes.[9] Related to these ligands, a dimeric form of AMND developed by Nakatani and coworkers[7c,d] is identified as having selective binding to a C–C mismatch in the surface plasmon resonance (SPR) platform. The C-selectivity of these ligands is rationalized by the protonation at the N1 position in the heterocycle to produce a hydrogen bonding surface fully complementary to that of C (Fig. 1A). On the other hand, the protonation at the N8 position in these ligands is also feasible, which is preferable for formation of a fully complementary hydrogen bonding to T (Fig. 1B). Therefore, such a possible protonation at both the N1 and N8 positions allows the binding to not only C but also T, which results in the moderate selectivity for C compared to T. Indeed, the difference in the binding affinity of AMND derivatives for C over T is only two- or three-fold,[5c] which is clearly insufficient for C/T discrimination.

In this work, we modified 2-amino-1,8-naphthyridine derivatives into a highly C-selective ligand for the AP site-containing DNA duplexes, for which we developed the substituted AMND derivative with an electron-withdrawing CF_3 group, CF_3-AMND (Fig. 1). CF_3-AMND was synthesized by reacting 2,6-diaminopyridine with 1,1,1-trifluoro-2,4-pentadione according to the literature (ESI†).[10] Here, the binding characteristics of CF_3-AMND to AP site-containing DNA duplexes were examined by UV-visible absorption and fluorescence measurements. The binding of CF_3-AMND was compared with the parent AMND in order to investigate the influence of the CF_3 group. In addition, we demonstrated that CF_3-AMND was applicable to the analysis of C-related single-base mutation in DNAs amplified by PCR. These results are discussed as the molecular basis for designing DNA-binding ligands suitable for the practical application to the accurate analysis of a single-base mutation.

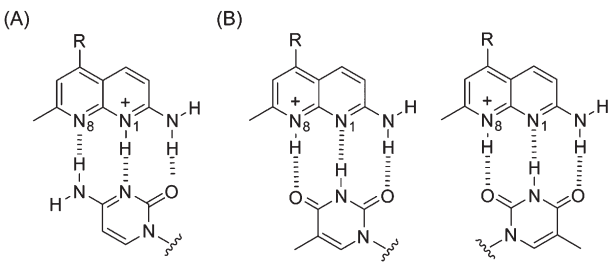

Fig. 1 Chemical structures of CF_3-AMND and AMND. Proposed binding modes of these ligands with (A) cytosine and (B) thymine (two possible patterns) are also shown.

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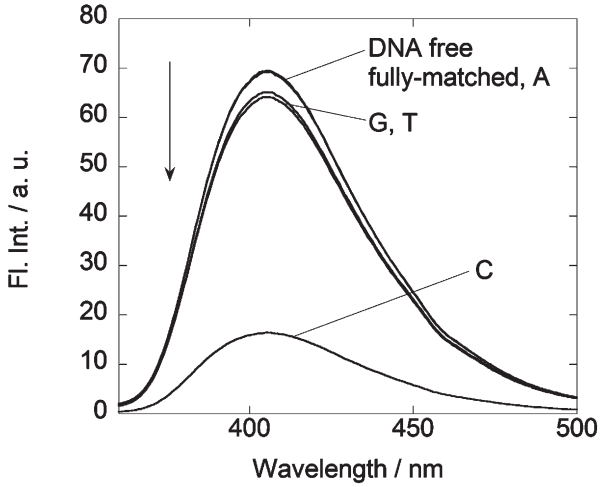

Fig. 2 Fluorescence response of CF_3-AMND (1.0 μM) to the target nucleobase in 21-mer AP site-containing DNA duplexes (5.0 μM; 5′-GCT CCT CTG GXG CCC TCG ACG-3′/3′-CGA GGA GAC CNC GGG AGC TGC-5′, X = AP site (dSpacer) or G, N = target nucleobase; G, C, A, or T) or normal (fully-matched) DNA duplex having no AP sites, measured in solution buffered to pH 7.0 (10 mM sodium cacodylate) containing 100 mM NaCl and 1.0 mM EDTA. Excitation, 346 nm. Temperature, 20 °C.

Fig. 2 shows the fluorescence responses of CF_3-AMND (1.0 μM) to 21-mer AP site-containing and fully-matched DNA duplexes (5.0 μM; 5′-GCT CCT CTG GXG CCC TCG ACG-3′/3′-CGA GGA GAC CNC GGG AGC TGC-5′, X = AP site (dSpacer) or G, N = target nucleobase; G, C, A, or T), measured at 20 °C in 10 mM sodium cacodylate buffer (pH 7.0) containing 100 mM NaCl and 1.0 mM EDTA. The addition of a fully-matched DNA duplex (X/N = G/C) having no AP sites does not cause any fluorescence response of CF_3-AMND, indicating negligible intercalative binding of CF_3-AMND. Also, almost no response is observed for AP site-containing DNA duplexes carrying the A target and only slight responses for G and T targets (Fig. 2). In contrast, significant fluorescence quenching is observed for C, where the fluorescence intensity at 403 nm decreases by as much as 75%. The high selectivity for C is consistent with the results observed for UV-visible absorption spectral changes (Fig. S1†). The absorption intensity of CF_3-AMND remarkably decreases upon addition of the AP site-containing DNA duplexes carrying C whereas the absorption changes are slight for fully-matched DNA duplexes and AP site-containing DNA duplexes carrying the G, A, or T target.

The binding affinity of CF_3-AMND to target nucleobases opposite the AP site was examined by fluorescence titration experiments. Fluorescence quenching response of CF_3-AMND (1.0 μM) was monitored upon addition of AP site-containing DNA duplexes in the concentration range from 0 to 7.0 μM (Fig. S2†). The resulting titration curves (Fig. 3A) could be well analyzed by non-linear least-squares regression based on a 1 : 1 binding model (ESI†) and the binding constant (K_{11}) for C was determined as 7.1 (± 0.2) × 10^5 M^{-1} (n = 3). Since the present concentration condition was not suitable for accurate determination of K_{11} for T, A, and G due to the weak binding, K_{11} values for these nucleobases were independently obtained in the high concentration conditions ([CF_3-AMND] = 10 μM, [DNA duplexes] = 0–70 μM). Significantly, the binding constant for C is found to be more than 50-fold larger than those for the other three nucleobases ($K_{11}/10^5$ M^{-1} (n = 3): T, 0.14 ± 0.016; G, 0.16 ± 0.029; A, <0.10). Such a difference in the binding constants for C over T (K_{11}(C)/K_{11}(T)) is much superior to those of the C-binding ligands developed previously.[5c,7c,d,9] These results clearly show that CF_3-AMND shows the strongest binding to C with high selectivity.

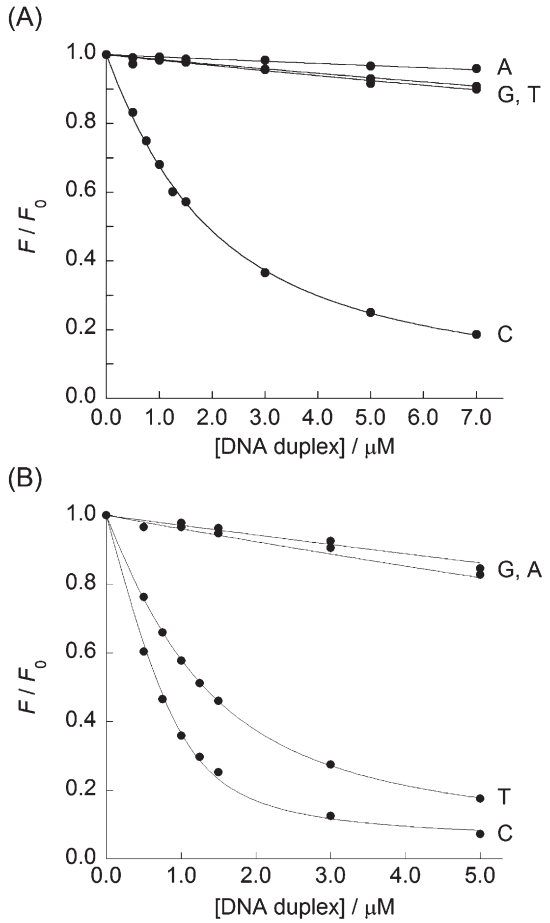

Fig. 3 Fluorescence titration curves for the binding of (A) CF_3-AMND (1.0 μM) and (B) AMND (1.0 μM) to the target nucleobases in 21-mer AP site-containing DNA duplexes. Other solution conditions were the same as those given in Fig. 2. F and F_0 denote the fluorescence intensities of CF_3-AMND in the presence and absence of DNA duplexes, respectively. Excitation: (A) 346 nm; (B) 350 nm. Analysis: (A) 403 nm; (B) 400 nm. Temperature, 20 °C.

The parent AMND shows stronger binding to C than CF_3-AMND for the same DNA sequence that was used in the fluorescence titration experiments for CF_3-AMND. The binding constant (K_{11}) obtained from the fluorescence titration experiments is 5.4 (± 0.5) × 10^6 M^{-1} (n = 3). This value is larger than that of CF_3-AMND, indicating a decrease in the binding affinity by attaching an electron-withdrawing CF_3 group to the parent AMND. On the other hand, AMND also shows strong binding to T as well as C (K_{11} for T; 1.3 (± 0.1) × 10^6 M^{-1}, n = 3), which leads to the relatively low selectivity of AMND for C over T (K_{11}(C)/K_{11}(T) = 4.2) compared to CF_3-AMND. Accordingly,

the CF_3 group is responsible for the high C-selectivity of CF_3-AMND.

Since 2-amino-1,8-naphthyridine derivatives can bind to the pyrimidine nucleobases in their protonated states,[5c,7c,d,9] we examined the pH dependence of the absorption spectra of CF_3-AMND to determine the pK_a value (Fig. S3†) and it was determined as 5.1. The decrease in pK_a compared to the parent AMND ($pK_a = 6.8$)[5c] is ascribed to the increase in the acidity of the aza-nitrogen of the AMND ring by attaching the CF_3 group. Thus, almost all of the CF_3-AMND molecules are in a neutral form at pH 7.0 in solution. In contrast, protonated CF_3-AMND binds to C and T at the AP site, as revealed by the examination of the salt dependence of the binding constants K_{11} based on Record's polyelectrolyte theory (ESI†).[11] The binding constants of CF_3-AMND for C and T were determined at different salt concentrations using fluorescence titration experiments. K_{11} values for C and T remarkably decrease as the salt concentration increases (Table S1†). The estimated apparent charges (Z) of CF_3-AMND were estimated as 1.47 and 1.02 when binding to C and T, respectively, as obtained from the linear regression analysis of a double logarithmic plot of K_{11} *versus* the Na^+ concentration (Fig. S4†). These results indicate that the protonation of the bound CF_3-AMND at pH 7.0 would be facile compared to the protonation in the free state, and this is consistent with the previously reported results.[5c,7c,d,9] As suggested in Fig. 1, CF_3-AMND binds to C and T in N1 and N8-protonated forms, respectively. Although further studies such as NMR analysis are required to examine the binding behaviors in detail, it is conceivable that the observed high selectivity of CF_3-AMND for C over T (Fig. 3A) arises from more favorable protonation at the N1 position than the N8 position due to the electron-withdrawing effect of the CF_3 group, which then enables the effective pseudo-base pairing with C through hydrogen bonding.

The results described above demonstrate the significant potential of CF_3-AMND as a highly selective ligand for C, although the binding affinity to C is relatively moderate ($K_{11} = 7.1 \times 10^5$ M^{-1}) compared to the methyl substituted AMND derivatives.[5c,8a,b] On the other hand, we found that the binding affinity of CF_3-AMND to C is remarkably increased under the acidic solution condition with pH lower than 7.0 (Fig. S5†). Significantly, the K_{11} value at pH 5.5 is 1.3 (± 0.1) × 10^7 M^{-1} ($n = 3$), which is two orders of magnitude larger than that at pH 7.0. The observed increase in the affinity can be explained by the increased amount of the N1 protonated CF_3-AMND species suitable for C-binding at pH 5.5. We did not do any of the experiments in the pH range lower than 5.5 considering the possible protonation of nucleobases. Importantly, high selectivity of CF_3-AMND for C is maintained at pH 5.5 (Fig. S6†), where the value of K_{11}(C)/K_{11}(T) is 58-fold. Moreover, CF_3-AMND shows high selective response for C compared to T at pH 5.5 regardless of the nucleobases flanking the AP sites in the DNA duplexes (Fig. S7†). Therefore, CF_3-AMND can work as an effective and versatile ligand for detection of the C-related single-base mutation in DNAs.

Finally, CF_3-AMND was applied to the analysis of single-base mutation in 107-mer DNAs (K-*ras* gene; codon 12, sense strand)[5c] amplified by asymmetric PCR (ESI†). Fig. 4 shows fluorescence responses of 0.1 μM CF_3-AMND to the amplified

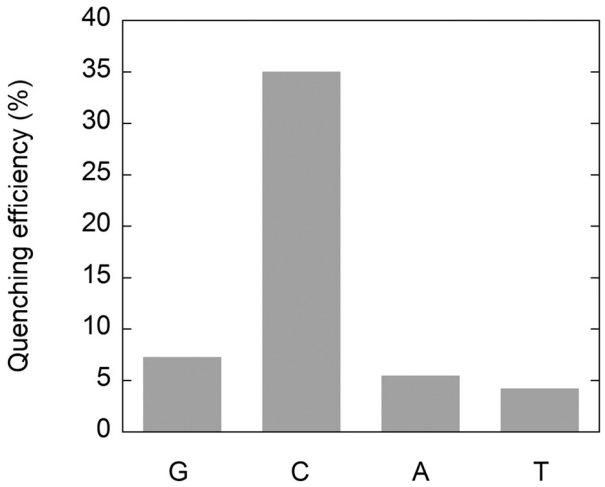

Fig. 4 Analysis of a single-base mutation in 107-mer DNAs (K-ras *gene*, codon 12, sense strand) amplified by PCR, measured in solutions buffered to pH 5.5 (100 mM sodium cacodylate) containing 2.0 μM AP site-containing probe DNA and 0.1 μM CF_3-AMND. Quenching efficiency (%) was calculated by $(F_0 - F)/F \times 100$, where F and F_0 denote the fluorescence intensities of CF_3-AMND in the presence and absence of DNA duplexes, respectively. Excitation, 346 nm. Analysis, 403 nm. Temperature, 5 °C.

DNAs carrying different target nucleobases measured at 5 °C in a solution buffered to pH 5.5 containing 2.0 μM AP site-containing probe DNA (5′-CCT ACG CCA XCA GCT CCA AC-3′, X = AP site (dSpacer)). As is clearly seen, CF_3-AMND exhibits significant fluorescence quenching for C-containing DNA sequences whereas moderate responses are observed for DNAs containing G, T, and A targets. Therefore, CF_3-AMND is applicable to highly selective detection of C compared to other nucleobases in PCR products, which will facilitate the accurate analysis of C-related single-base mutation in practical use.

In summary, we reported that CF_3-AMND shows highly selective binding to C in AP site-containing DNA duplexes. The introduction of a CF_3 substituent into the parent AMND significantly improved the binding selectivity for C. We expect that high C-selectivity of CF_3-AMND will be able to provide accurate analysis of C-related single-base mutation in DNAs. We also expect that CF_3-AMND will function as the recognition unit with high C-selectivity for the design of the conjugates having emissive[12] and ratiometric signaling functions.[13] On the other hand, we noted that the binding affinity of CF_3-AMND to C is relatively moderate at the physiological pH condition, and further efforts to improve the affinity are thus required to enhance the practicability towards gene analysis. We are now undertaking further studies in this direction.

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