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2-Aminopurine-Modified Abasic-Site-Containing Duplex DNA for Highly Selective Detection of Theophylline

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Aptamers (short nucleic acid-binding species) have emerged as promising candidates for molecular recognition events on account of their significant ability to bind a large number of ligands.[1−4] High affinities and specificities of aptamers toward target ligands are generally achieved by a combination of molecular-shape complementarity, hydrogen bonding, and stacking interactions.[1] Such binding events typically involve ligand-induced structural changes in the aptamers, resulting in the formation of unique secondary structures responsible for the ligand binding, of which the internal-loop and stem−loop structures are representative of active sites for binding events.

On the other hand, we have recently reported a relatively simple class of DNA duplex aptamers[5] in which an abasic site[6] was utilized as an active cavity for binding events (Figure 1A). While a naturally

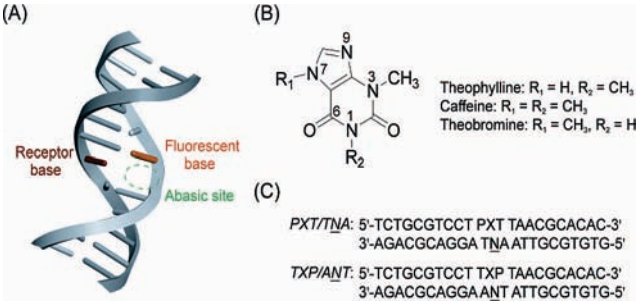

Figure 1. (A) Illustration of a fluorescent signaling aptamer based on abasic-site-containing duplex DNA. (B) Chemical structures of theophylline and its derivatives. (C) Names and sequences of duplex DNAs examined in this study: P = 2-aminopurine, X = abasic site (Spacer-C3, a propyl linker), and N = receptor base (G, C, A, or T).

occurring abasic site is one of the most common forms of DNA damage,[6] we have incorporated such lesion sites, but in a chemically stable form, into the duplex. The nucleotides constituting the active cavity were then optimized to promote ligand binding selectively and strongly by pseudobase pairing with an intrahelical receptor nucleobase and by stacking with nucleobases flanking the abasic site. Indeed, from the examination of the receptor nucleobases, the length of the duplexes, and the nucleobases flanking the abasic site, a 23-mer duplex containing thymine as the receptor base was found to selectively bind to riboflavin over flavin mononucleotide and flavin adenine dinucleotide with a dissociation constant of 1.9 μM.[5] The observed binding affinity up to the micromolar range was almost comparable with binding affinities of RNA aptamers developed earlier for flavin derivatives.[3] In addition to such promising binding functions, the abasic-site-based DNA aptamer may have some advantages, such as its easy, low-cost synthesis and higher chemical stability, as compared with typical RNA aptamers.

In this work, the abasic-site-based DNA aptamer is developed into a signaling aptamer for which a fluorescent adenine analogue, 2-aminopurine (2-AP), is incorporated into the duplex to flank the abasic site (Figure 1A). The fluorescence of 2-AP is very sensitive to its environment, and therefore, 2-AP has been extensively used as a probe for local structure and dynamics in DNA.[7] In the present aptamer, less-stacked 2-AP adjacent to the abasic site becomes more stacked with neighboring bases and/or ligands upon binding, and the stacking causes quenching of 2-AP. Herein, we report on such a fluorescent signaling aptamer that targets theophylline (Figure 1B), a drug for diseases such as bronchial asthma and bradycardia.[8]

The design strategy utilized here is relatively simple, focusing on the examination of (i) the nucleobases flanking 2-AP, (ii) the position of 2-AP, and (iii) the receptor nucleobases opposite the abasic site. It has been demonstrated that 2-AP exhibits quenching when stacked in duplex DNA and that guanine causes the strongest quenching of 2-AP.[7a,b] This indicates that AT base pair would be suitable as the nearest-neighbor base in order to minimize the quenching of 2-AP in the uncomplexed duplex. It has also been reported that the fluorescence intensity of 2-AP adjacent to the abasic site in duplex DNA depends on its relative position (5′ or 3′),[7c] indicating that the difference in position of 2-AP would be crucial for effective signaling. From these considerations, we prepared two series of 23-mer duplexes having 2-AP on the 5′ or 3′ side of the abasic site and a different nucleobase (G, C, A, or T) opposite the abasic site (Figure 1C). In these duplexes, 2-AP is flanked by thymine, and the sequence of the other region is identical to that of the 23-mer duplex previously reported for riboflavin binding.[5]

First, fluorescence spectra of duplexes having 2-AP on the 5′ side of the abasic site [5.0 μM, PXT/TNA, N = receptor base (G, C, A, or T)] were examined in the presence or absence of theophylline (200 μM) in solutions buffered at pH 7.0 (5 °C, I = 0.11 M). For all of the duplexes, emission due to 2-AP with a maximum at 367 nm was observed, but the fluorescence intensity and response were strongly dependent on the receptor base opposite the abasic site (Figure S1 in the Supporting Information). PXT/TGA, with guanine as the receptor base, exhibited the weakest emission, and almost no response was observed even in the presence of 40 equiv of theophylline. Similarly, the response to theophylline was only moderate for PXT/TAA and PXT/TTA. On the other hand, effective quenching was observed for PXT/TCA having cytosine as the receptor base (also see Figure 2A), and uncomplexed PXT/TCA exhibited the strongest emission among these duplexes. It is therefore highly likely that theophylline binding takes place when cytosine is placed opposite the abasic site, and this also benefits minimum quenching of 2-AP in the uncomplexed duplex.

Similar results were obtained for duplexes having 2-AP on the 3′ side of the abasic site [TXP/ANT, N = receptor (G, C, A, or T)]. Again, an effective quenching response to theophylline was obtained for TXP/ACT having cytosine as a receptor base, and its emission (λ_{max} = 367 nm) was the strongest among the four duplexes in the uncomplexed form (Figure S2 in the Supporting

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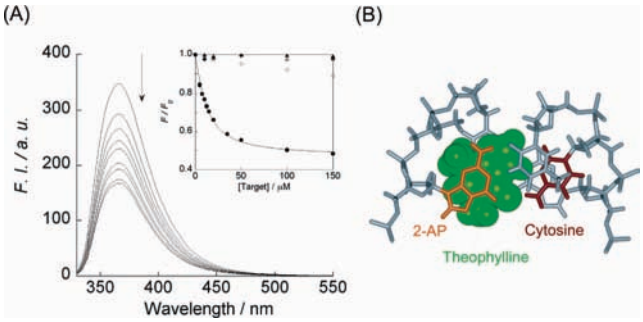

Figure 2. (A) Fluorescence response of PXT/TCA (10 μM) to theophylline (0−150 μM) in solutions buffered at pH 7.0 (10 mM sodium cacodylate) containing 100 mM NaCl and 1.0 mM EDTA. Excitation was at 320 nm and $T = 5$ °C. Inset: responses of PXT/TCA (10 μM) to (●) theophylline, (△) caffeine, (+) theobromine, (■) uric acid, (▲) creatinine, and (◆) glucose. F and F_0 denote the fluorescence intensities (367 nm) in the presence and absence of ligands, respectively. (B) Possible binding mode of the theophylline−cytosine complex in PXT/TCA, obtained using MacroModel version 9.0. A CPK model of theophylline is shown in green, 2-aminopurine (2-AP) in yellow, and cytosine in red. Also see Figure S3 in the Supporting Information.

Information). The observed quenching up of to 45% obtained for TXP/ACT is almost comparable to the response obtained for PXT/TCA having 2-AP on the 5′ side (50%). However, the intensity of uncomplexed TXP/ACT is significantly lower than that of PXT/TCA (the ratio of the fluorescence intensities at 367 nm is $F_{\mathrm{TXP/ACT}}/F_{\mathrm{PXT/TCA}} = 0.45$), which is a disadvantage for sensor applications. We therefore qualified PXT/TCA as an aptamer for further investigation.

An examination of the binding affinity and selectivity reveals a promising feature of PXT/TCA for theophylline detection. As shown in Figure 2A, the response of PXT/TCA to theophylline is concentration-dependent, which is explained by 1:1 binding with a dissociation constant K_d of 10 ± 1.0 μM (inset of Figure 2A, $n = 3$). Significantly, PXT/TCA exhibits almost no response to either caffeine or theobromine (inset of Figure 2A), each of which differs from theophylline only by a single methyl group attached to the xanthine ring (Figure 1B). In addition, the responses to other substrates such as uric acid and creatinine, present in serum, are only moderate or negligible (Figure 2A). Apparently, PXT/TCA does work as a signaling aptamer with a high selectivity for theophylline.

The all-or-none binding to theophylline over caffeine and theobromine is a distinguishing feature and is like that of the theophylline-binding RNA aptamer[4a,b] developed by the SELEX process. This 33-mer RNA aptamer has two internal loops that form the binding site and is able to discriminate caffeine on the basis of a 10^4-fold difference in the binding affinity [K_d (μM): theophylline, 0.32; caffeine, 3500; theobromine, >500]. The selective binding is explained by the formation of two hydrogen bonds between theophylline and cytosine, for which the N-7 hydrogen and C-6 keto oxygen of theophylline are critical. In the case of a 13-mer RNA aptamer with a relatively moderate binding selectivity [K_d (μM): theophylline, 13; caffeine, 590),[4c] theophylline forms two hydrogen bonds to cytosine and uracil, where the N-7 hydrogen and N-9 nitrogen are involved in the binding.

In the present case, it is feasible that theophylline−cytosine binding is promoted by two hydrogen bonds involving the N-7 hydrogen and C-6 keto oxygen of theophylline (Figure 2B and Figure S3 in the Supporting Information), similar to the binding in the 33-mer RNA aptamer.[4a,b] This explains the loss of binding affinity for caffeine and theobromine, which have an additional methyl group at the N-7 position. Also, this binding mode would allow effective stacking with the adjacent 2-AP (Figure 2B), resulting in quenching upon theophylline binding.

The binding affinity of PXT/TCA is only moderate compared with the 33-mer RNA aptamer,[4a,b] but it is strong enough for the detection of theophylline in serum samples (Figure S4 in the Supporting Information). Here, a horse serum control sample containing theophylline (5 μL) was analyzed after dilution with a buffer solution (45 μL, pH 7.0) that contained PXT/TCA. A linear response to theophylline was obtained in the concentration range from 5 to 20 μM, which corresponds to the concentration range from 50 to 200 μM in the original serum samples. PXT/TCA is thus applicable to the monitoring of serum theophylline concentrations in the therapeutic range (60−100 μM),[8] and the analysis requires no further purification of serum samples such as deproteination.

In summary, we have successfully developed a fluorescent signaling aptamer based on abasic-site-containing duplex DNA that has a useful sensitivity for the bronchodilator theophylline. The approach described here is straightforward, and further modification of the binding cavity by synthetic receptor and/or fluorescent nucleobases would be promising for the design of this class of DNA aptamers for a variety of small ligands. We are now undertaking further studies in this direction.

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Supporting Information Available: Fluorescence responses to theophylline of duplexes having 2-AP on the 5′ or 3′ side of the abasic site, possible binding mode of the theophylline−cytosine complex, and analysis of theophylline in serum samples. This material is available free of charge via the Internet at http://pubs.acs.org.

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