RNA Structures

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Strong and Selective Binding of Amiloride to an Abasic Site in RNA Duplexes: Thermodynamic Characterization and MicroRNA Detection**

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Small ligands that can bind to nucleic acids have been of great interest because of their promising abilities as therapeutical candidates to modulate or inhibit the crucial functions of nucleic acids.[1] These ligands have also been used as powerful tools for the detection of nucleic acids in solutions as well as in various platforms such as gels[2] and microarrays.[3] The non-covalent binding modes of ligands with DNAs are broadly divided into two categories, that is, intercalation and minor groove binding.[4] Intercalation is the insertion of a planar aromatic ligand between base pairs of DNA duplexes, whereas in minor groove binding a crescent-shaped ligand fits into the minor groove of the DNA duplexes. Wilson et al.[5] systematically investigated the binding abilities of various DNA-binding ligands to RNA duplexes to obtain the molecular basis for the design of drugs targeting RNAs. Whereas minor groove binders generally exhibit neglible binding for RNA duplexes because of the structural difference between B-formed DNA and A-formed RNA duplexes, many intercalators can bind to RNA duplexes. However, nearly all intercalators show more favorable binding to DNA duplexes than RNA duplexes.[5] The properties of DNA-binding ligands do not directly lead to the scaffold design for RNA-binding ligands suitable for potential applications in analytical, biological, and medicinal fields.[1b,6] On the other hand, aminoglycoside antibiotics have been used as a typical RNA-binding scaffold because of their high binding selectivity for A-formed RNAs with dissociation constants in the nanomolar range.[7] However, it has been recognized that aminoglycosides show promiscuous binding to various RNA targets because of their electrostatically driven binding mode and their conformational adaptability.[7c,8] The low binding selectivity has limited the use of aminoglycosides in various application fields, though some attempts were reported to increase the selectivity for specific target RNA structures by developing conformationally constrained aminoglycoside derivatives.[8b,c]

We have recently found a series of small ligands that can bind specifically to an abasic site (AP site) in DNA duplexes with fluorescence signaling.[9] In contrast to intercalators or minor groove binders,[10,11] these ligands can bind selectively to the abasic site in DNA duplexes, in which the binding event is strongly and selectively promoted by a pseudo-base pairing with an intrahelical nucleotide opposite the AP site and by stacking with nucleotides flanking the AP site. We have shown that these ligands are applicable to the typing of single-nucleotide polymorphisms.[9] Recently, these ligands have been shown to work as a site-specific staining agent for the design of label-free aptamer[12a–c] and molecular beacon systems.[12d]

Here, we describe the significantly stronger binding of amiloride (Figure 1)[9d] to the AP site in RNA duplexes relative to DNA duplexes. While AP sites are one of the well-known DNA lesions,[13] AP sites have been also found to naturally occur in some RNAs and the chemical and biological properties of such AP sites in RNAs have recently gained much attention for providing better understanding of the cellular repair system.[14] AP site-containing RNA duplex (AP-RNA) examined in this study possesses a uracil nucleotide opposite the AP site (spacer C3; a propyl residue; Figure 1), so that amiloride is expected to bind strongly to this uracil nucleotide in the RNA duplex by formation of fully complementary hydrogen bonding through two possible binding modes (Figure 1B). The binding characteristics of

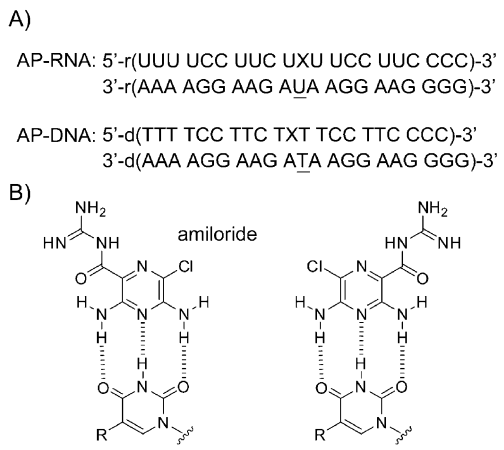

Figure 1. A) Sequences of 21-mer AP site-containing RNA duplexes (AP-RNA) and DNA duplexes (AP-DNA): X = AP site (spacer C3, a propyl residue), U and T = nucleotide opposite an AP site. B) Possible binding mode of amiloride with uracil ($R = CH_3$) and thymine ($R = H$) nucleotide opposite an AP site.

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amiloride to AP-RNA were examined by fluorescence, circular dichroism (CD), and isothermal titration calorimetry (ITC) measurements, and the obtained data were compared with the corresponding AP site-containing DNA duplexes (AP-DNA). Moreover, we demonstrated that amiloride is applicable to the detection of microRNAs (miRNAs) that have been associated with various diseases,[15] based on the sensitive fluorescence response of the amiloride binding to the AP site in RNA duplexes.

Figure 2A shows the fluorescence spectra of amiloride (100 nM) in the absence and presence of AP-RNA (100 nM), measured at 20°C in 10 mM sodium cacodylate solution (pH 7.0) containing 100 mM NaCl and 1.0 mM EDTA. In the absence of AP-RNA, amiloride exhibits emission with a maximum at 415 nm (curve a). Addition of AP-RNA causes a significant enhancement in the fluorescence intensity of amiloride (curve b) whereas no response is observed for a fully-matched RNA duplex having no AP sites (curve c: 5′-r(UUU UCC UUC UAU UCC UUC CCC)-3′/3′-r(AAA AGG AAG AUA AGG AAG GGG)-5′) as well as single-stranded RNAs consisting of AP-RNA (curves d and e). These results indicate highly selective binding of amiloride to the AP site of AP-RNA.

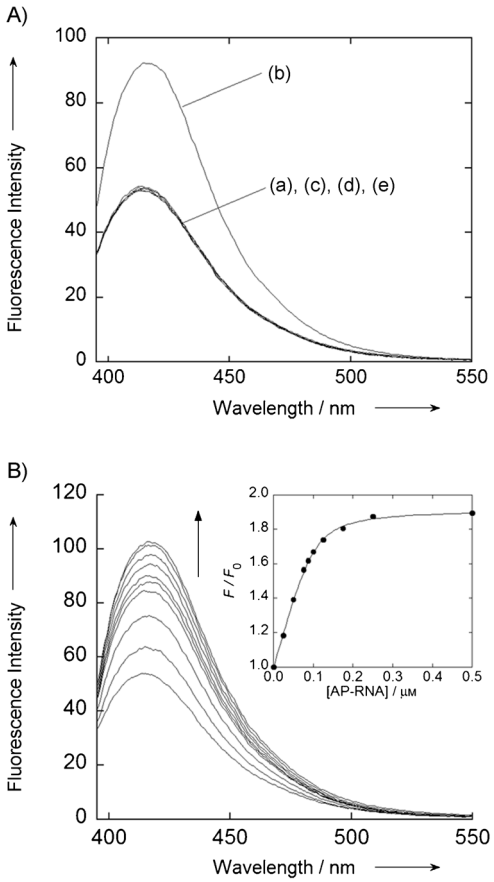

Figure 2. A) Fluorescence spectra of amiloride (100 nM) in the a) absence and b) presence of AP-RNA (100 nM), c) normal RNA duplex having no AP sites, d) one RNA single-strand of AP-RNA, (5′-r(UUU UCC UUC U*X*U UCC UUC CCC)-3′, *X* = AP site (spacer C3), e) another RNA single strand of AP-RNA (5′-r(GGG GAA GGA AUA GAA GGA AAA)-3′). Sample solutions were buffered to pH 7.0 with 10 mM sodium cacodylate, containing 100 mM NaCl and 1.0 mM EDTA. Excitation, 381 nm. Temperature, 20°C. B) Fluorescence response of amiloride (100 nM) to AP-RNA (0–500 nM). Inset: non-linear 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 AP-RNA, respectively.

The binding affinity of amiloride to AP-RNA was examined by the fluorescence titration experiments. As is shown in Figure 2B, fluorescence response of amiloride to AP-RNA shows the concentration dependence. The resulting titration curve (inset of Figure 2B) was well fitted by a 1:1 binding isotherm, which gives a dissociation constant K_d of (9.5 ± 0.66) nM ($n = 3$). Significantly, the binding affinity of amiloride to AP-RNA is stronger than that of typical aminoglycoside derivatives, such as paromycin ($K_d = 200$ nM, 150 mM NaCl, pH 7.4) and neomycin ($K_d = 19$ nM, 150 mM NaCl, pH 7.4) for the A-site of 16S rRNA,[16] one of the most studied models for RNA–ligand interactions. Amiloride binding to the AP site in the RNA duplexes strongly depends on the nucleotide opposite an AP site (see Table S2 in the Supporting information). The binding affinity for cytosine opposite an AP site is 28-times weaker ($K_d = 267 \pm 70.9$ nM, $n = 3$) than that for uracil whereas the K_d values for guanine and adenine are too small to be determined under the present conditions. Such a binding selectivity of amiloride for uracil over other nucleotides at an AP site is rationalized by the pseudo-base pairing through the complementary hydrogen bonding (Figure 1B). Uracil selectivity of amiloride is also observed when a tetrahydrofuranyl residue (dSpacer) was used as an AP site instad of spacer C3, but the binding affinity for uracil or cytosine opposite the dSpacer is one order of magnitude smaller compared to spacer C3 (see Table S2 in the Supporting Information). The decrease in the affinity is likely to arise from the steric hidrance because of the relatively small size of the dSpacer AP site for amiloride binding.

Amiloride was found to bind more strongly to AP-RNA than AP-DNA. Similar to AP-RNA, the fluorescence intensity of amiloride increases upon addition of AP-DNA, which shows the amiloride binding to uracil opposite the AP site in the DNA duplex (see Figure S1 in the Supporting Information). It was found that the K_d value for AP-DNA (740 ± 84 nM, $n = 3$) obtained from fluorescence titration experiments is much larger compared to AP-RNA and that amiloride binding to AP-RNA is two orders of magnitude superior to AP-DNA. Such a remarkable preference of amiloride binding to RNA relative to DNA is quite characteristic compared to typical small DNA-binding ligands.[1b,5,6]

To understand the stronger binding of amiloride for AP-RNA over AP-DNA, we examined the conformational changes of AP-RNA upon binding of amiloride by circular dichroism (CD) experiments (see Figure S2 in the Supporting information). AP-RNA exhibits the CD spectrum characteristic of the A-form, which is quite similar to the normal RNA duplex having no AP sites (see Figure S2A in the Supporting Information). The addition of amiloride causes little change in the CD spectrum, indicating that the resulting amiloride/AP-RNA complex remains in the A-form without any drastic conformational change. As for AP-DNA, the observed CD

spectrum indicates the B-form structure and it is not altered upon amiloride binding (see Figure S2B in the Supporting Information). On the other hand, the A-form geometry in the nucleic acids allows more extensive base–base overlap and provides more efficient stacking interactions of nucleotides than the B-form genometry.[17] It is therefore likely that such efficient stacking interactions between amiloride and the nucleotides flanking the AP site play a considerable role in the more significant binding affinity of amiloride to AP-RNA over AP-DNA.

Thermodynamic characterization by isothermal titration calorimetry (ITC) supports the efficient stacking interaction at the AP site of AP-RNA. From the analysis of the ITC data (see Figure S3 in the Supporting Information), we obtained the thermodynamic parameters for the amiloride binding to AP-RNA and compared them to those of AP-DNA (Table 1).

Table 1: Thermodynamic parameters for the binding of amiloride to AP-RNA and AP-DNA.[a]

	AP-RNA	AP-DNA
K_d [nM][b]	9.5 (±0.7)	740 (±84)
ΔG_{obs} [kcal mol^{-1}][b]	−10.8 (±0.1)	−8.2 (±0.1)
ΔH_{obs} [kcal mol^{-1}][b]	−16.5 (±0.4)	−13.9 (±0.4)
$T\Delta S_{obs}$ [kcal mol^{-1}][b]	−5.7 (±0.4)	−5.7 (±0.4)

[a] K_d is the dissociation constant with 1:1 binding, obtained from the fluorescence titration experiments (Figure 2B and Figure S1 in the Supporting Information). ΔG_{obs} is the observed binding free-energy calculated from $\Delta G_{obs} = -RT\ln K_{11}$ (K_{11} (the binding affinity) $= 1/K_d$). ΔH_{obs} was directly determined by isothermal titration calorimetry (ITC; Figure 4). $T\Delta S_{obs}$ was calculated from $T\Delta S_{obs} = \Delta H_{obs} - \Delta G_{obs}$. [NaCl] = 100 mM, [EDTA] = 1.0 mM, [sodium cacodylate] = 10 mM, pH 7.0, and $T = 20$ °C. [b] Errors are the standard deviations obtained from three independent experiments.

While the binding events in both AP-RNA and AP-DNA are enthalpically driven, the observed binding enthalpy (ΔH_{obs}) for AP-RNA is more favorable by 2.6 kcal mol^{-1} compared to that AP-DNA. We also found that the binding entropy ($T\Delta S_{obs}$) between AP-RNA and AP-DNA is comparable. Accordingly, the observed favorable ΔH_{obs} for AP-RNA over AP-DNA is responsible for the increase in the observed binding free-energy (ΔG_{obs}) of amiloride. Since the change of the free-energy of the stacking interactions in nucleic acids is dominated by an enthalpy term (ΔH),[18] this favorable ΔH_{obs} arises from the more efficient stacking interactions between amiloride and the neighboring nucleotides of the AP site in A-formed AP-RNA compared to B-formed AP-DNA.

Figure 3 shows the energetically stabilized structures of amiloride/AP-RNA and amiloride/AP-DNA complexes obtained by molecular modelling simulation (see Figure S4 in the Supporting Information). For binding to AP-RNA (Figure 3A), amiloride is well stacked with not only the 3′-flanking uracil nucleotide of the AP site in the RNA strand but also the neighboring adenine nucleotide in another RNA strand of AP-RNA. Such stacking behavior with the neighboring nucleotides in the RNA duplex was also proposed for a small ligand that could bind to the cytosine bulge in RNA duplexes.[19] On the other hand, amiloride seems to stack only

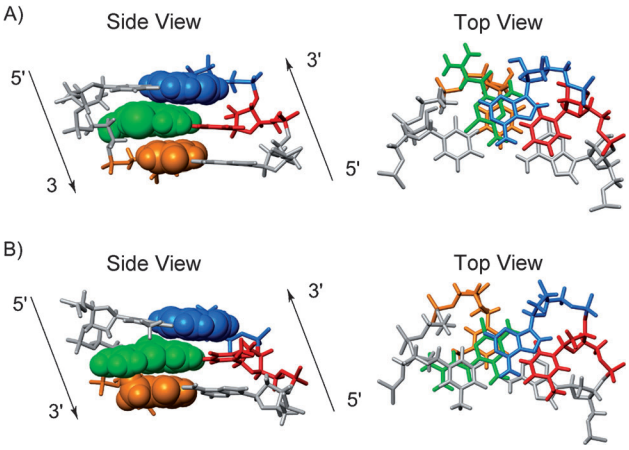

Figure 3. Molecular modeling of A) the amiloride/AP-RNA complex and B) the amiloride/AP-DNA complex, obtained by MacroModel version 9.0 (Amber* force field and the GB/SA water model). Green: amiloride. Red: the nucleotide opposite an AP site. Orange: 3′-flanking A) uracil nucleotides to the AP site or B) thymine nucleotides to the AP site. Blue: the neighboring adenine nucleotides. In the side view of these structures, amiloride, the 3′-flanking nucleotide to the AP site, and the neighboring nucleotide are shown in the CPK model for clarity of the stacking interations in these complexes.

with the 3′-flanking thymine nucleotide of the AP site in the complex with AP-DNA (Figure 3B). While further structural studies such as NMR analysis are needed to clarify the observed stacking interactions, the favorable stacking interactions of amiloride in AP-RNA are likely responsible for the significant affinity to AP-RNA.

Finally, amiloride is applied to the detection of miRNAs that have recently been shown to be linked with the onset of cancer and diseases.[15,20] We chose three kinds of let-7 family members including let-7a, let-7f, and let-7g as target miRNAs (Figure 4A) because of their relationship with cell develop-

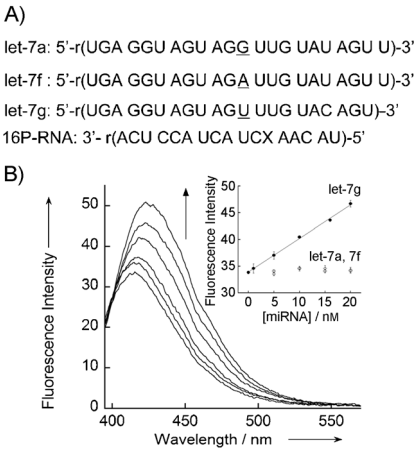

Figure 4. A) Sequences of let-7a, 7f, and 7g used as target miRNAs and of a 16-mer AP site-containing RNA probe (16P-RNA): X denotes the AP site (spacer C3). B) Fluorescence response of amiloride (50 nM) to the let-7g/16P-RNA duplex (0–20 nM). Inset: Changes in the fluorescence intensity at 415 nm of amiloride upon addition of let-7a (○), 7f (◇), and 7g (●). Error bars are the standard deviations obtained from three independent experiments.

ment and human cancer.[21] For the analysis of these miRNAs, a 16-mer AP site-containing RNA probe (16P-RNA) was prepared (Figure 4A) and the hybridization between 16P-RNA and let-7a, 7f, and 7g allowed the formation of RNA duplexes containing guanine, adenine, and uracil (Figure 4A; underlined nucleotide) opposite the AP site, respectively. Amiloride exhibits a significant fluorescence response to let-7g (Figure 4B) whereas almost no response was observed for guanine-containing let-7a and adenine-containing let-7f (Inset, Figure 4B). The highly selective response of amiloride for let-7g arises from the amiloride binding to uracil in let-7g/16P-RNA duplex (see Figure S5 in the Supporting Information), where the dissociation constant K_d to the duplex reaches (8.8 ± 0.80) nM ($n = 3$). Apparently, amiloride is applicable to the selective detection of let-7g over let-7a and let-7f. The limit of detection (LOD) for let-7g was determined as 1.2 nM (Figure S6), corresponding to 59 fmol in a 50 μL. While the present experiments were performed using the synthetic miRNAs, this value of LOD is comparable to that of northern blotting method used as a standard method for miRNA detection.[22,23] To make the present method more suitable for practical application,[24] further efforts are needed to improve the sensitivity and selectivity for target miRNAs, for which the binding affinity, selectivity, and binding-induced response of AP site-binding ligands should be improved; however, it is noteworthy that the combination of amiloride with a AP site-containing RNA probe is very applicable to the miRNA detection.

In summary, we revealed that amiloride is able to bind strongly and selectively to an AP site in RNA duplexes with fluorescence signaling and that its affinity to RNA is significantly stronger than that to DNA. Such a significant binding preference of amiloride for RNA over DNA is of great interest for the design of RNA-binding ligands considering the fact that most DNA-binding ligands display little binding selectivity between RNAs and DNAs.[1b,5,6] Moreover, amiloride was applicable to the fluorescence detection of miRNAs. We expect that the results will offer valuable insights for the design of RNA-selective binding ligands and for the development of a new ligand-based strategy for miRNA detection. We also expect that amiloride will be a useful candidate to explore unknown biological roles of AP sites present in RNAs.[14] We are now undertaking further studies in these directions.

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