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Bioorganic & Medicinal Chemistry Letters 15 (2005) 827–831
Bioorganic & Medicinal Chemistry Letters

Specific recognition of napthyridine-based ligands toward guanine-containing bulges in RNA duplexes and RNA–DNA heteroduplexes

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Received 8 September 2004; revised 13 October 2004; accepted 15 October 2004
Available online 13 December 2004

Abstract—Mismatched bulges in nucleic acid constructs are important in the recognition event between biological molecules. Herein, it is observed that napthyridine dimer **2** is able to specifically bind G–G mismatches in all nucleic acid constructs comprising of RNA–RNA, RNA–DNA and DNA–DNA duplexes. However, the binding affinity of **2** is strongest toward DNA duplex, followed by RNA–DNA heteroduplex and RNA duplex being the weakest binding partner. Nonetheless, this binding behavior suggests that the binding process primarily occurs between the guanine base pairs and the napthyridine moiety, and is independent of the tertiary structure of the nucleic acid duplexes.
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1. Introduction

Specific recognition of mismatched bulges in nucleic acid stem loop are important toward many biological processes such as identifying genetic mutations, developing potential antagonists, and detecting single nucleotide polymorphism (SNP).[1–4] Developing ligands that would specifically bind to mismatched bulges in RNA is of interest as a majority of RNA-ligand recognition processes have been observed to occur through the RNA stem loop.[5–9] Examples include binding of aminoglycoside antibiotics to a 7 nucleotide (nt) stem loop of the A-site 16S rRNA[10] and the recognition of both HIV Tat and Rev peptides to mismatched nucleotide stem loop of their respective TAR and RRE RNA constructs.[11] The HIV-1 RRE IIB RNA region is of particular interest as it is an essential region for the successful transcription of the HIV-1 genome.[12–15] It is also the site in which the arginine-rich Rev peptide binds, the resulting complex is then transported either through the nuclear membrane or the nuclear pore complex into the cytoplasm, resulting in the RNA being eventually translated into the viral protein.[15–17]

The Rev-RRE interaction is previously demonstrated to be modular, as these constructs have successfully been dissected into smaller fragments without affecting their funtionalities.[18,19] Investigation of these fragments indicate the 5 nt stem loop of the HIV RRE IIB RNA construct was the binding site for the HIV Rev peptide. In this communication, mutant nucleic acid constructs of the HIV RRE IIB RNA have been generated for our binding studies, primarily because three nts within the binding stem loop are unpaired guanine bases. To target these unpaired guanine bases, we turn our attention to a series of novel napthyridine-based ligands that was recently reported by Nakatani and co-workers.[20–24] Specifically, we focused on napthyridine monomer **1** and dimer **2** (Fig. 1) as both ligands were observed to specifically recognize either a single guanine bulge or guanine–guanine (G–G) mismatch in DNA.[20–22] In addition, the binding selectivity of both **1** and **2** are remarkable as binding to other nucleotides or mismatches was not observed.[20–22]

Herein, binding studies of **1** and **2** to guanine mismatches in HIV-1 RRE IIB RNA duplex, RNA–DNA heteroduplex, and DNA duplex are reported. Briefly, results obtained indicate that napthyridine dimer **1** is

Keywords: Napthyridine; Fluorescence; Nucleic acids; Circular dichroism; DNA melt.
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0960-894X/$ - see front matter Published by Elsevier Ltd.
doi:10.1016/j.bmcl.2004.10.059

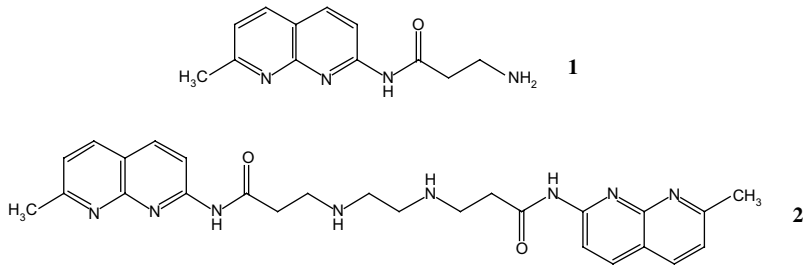

Figure 1. Structures of the molecules tested: napthyridine monomer **1** and napthyridine dimer **2**.

able to bind specifically to G–G mismatches in RNA constructs, but the binding is indiscriminate as G–G mismatches within duplexes of RNA–DNA and DNA–DNA are also able to bind to both ligands **1** and **2**. However, the binding affinity was slightly lower for RNA duplex and RNA–DNA heteroduplex compared to DNA–DNA duplex.

2. Sample preparation and fluorescence measurement

We have recently observed that a single hairpin HIV TAR RNA construct can be converted to a TAR RNA duplex construct without drastically affecting its Tat peptide binding ability, provided that appropriate stabilizing G–C base pairs were inserted at the duplex ends.[25] In this study, we extend this observation to a HIV RRE IIB RNA construct, in which the 4 nt hairpin loop (GCAA) and the single nt bulge (A) of the HIV RRE IIB RNA was eliminated. Together with modifications on the 5 nt stem loop, mutants of the duplex nucleic acid constructs were prepared (secondary structures of all mutants are collectively shown in Fig. 2). All the resulting mutant constructs have either one of its DNA or RNA strand labeled with a fluorescein moiety at the 3′ end, that is, *Fl*-D1 or *Fl*-R1, such that the resulting duplex binding behavior can easily be studied through fluorescence measurements. Using a 'mix and match' approach, different duplex constructs can be easily generated. To ensure that dimer **2** is binding specifically to the G–G mismatched stem loop, constructs that do not possess the G–G mismatched stem loop were also prepared (constructs **E** and **F**).

Both napthyridine monomer **1** and dimer **2** were synthesized according to previously published procedures.[22,24]

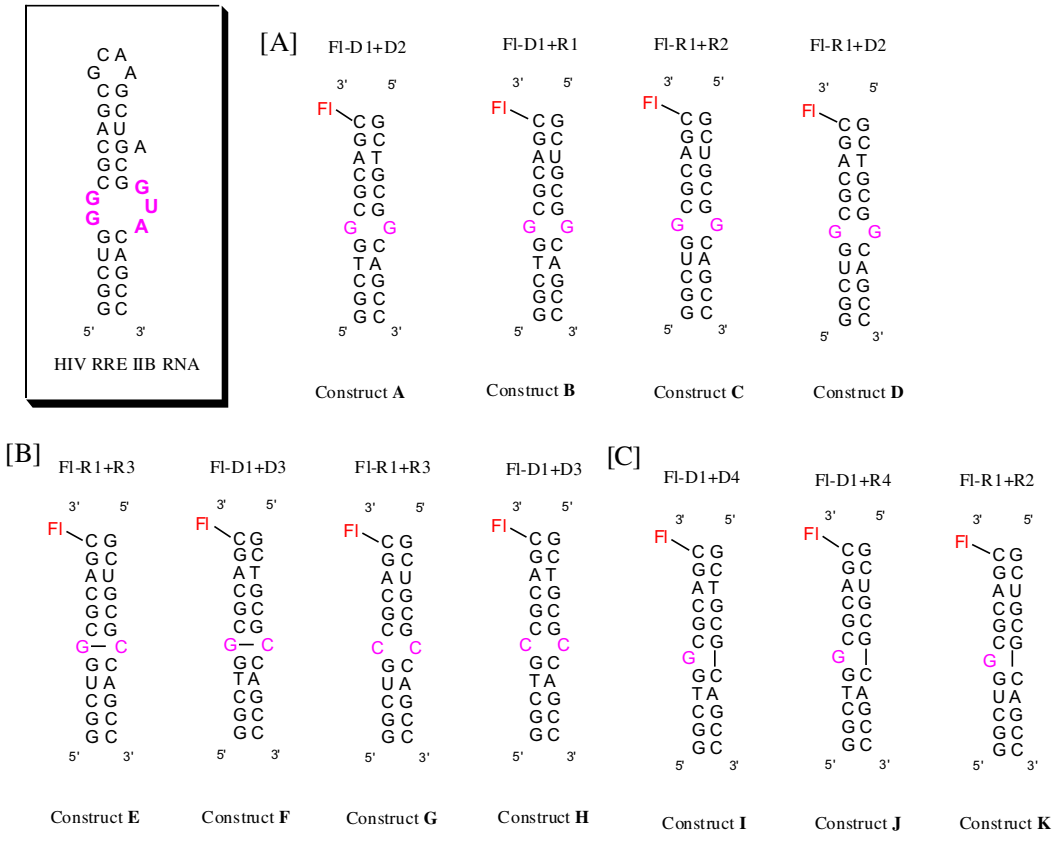

Figure 2. Secondary structures of the stem loop mutant HIV RRE IIB nucleic acid constructs that contained: [A] G–G mismatches (constructs **A** to **D**), [B] no mismatches (constructs **E** and **F**) and C–C mismatches (constructs **G** and **H**), [C] only a single G-bulge (constructs **I** to **K**). Note that all the constructs are fluorescein-labeled (*Fl*-) at the 3′-end of either D1 or R1 strands.

The RRE mutant constructs **A** to **K** was prepared through hybridizing appropriate gel-purified RNA or DNA strands (Dharmacon Inc., Lafayette, CO). The annealing process for the various nucleic acid strands was performed at 95 °C for 4 min and cooling to 25 °C over 30 min in an Eppendorf thermal cycler. Binding affinities of fluorescein-labeled duplex mutant RRE constructs **A** to **K** to either ligand **1** or **2** were analyzed by the fluorescence binding method as described previously.[26–28] Upon titration of 10 nM of fluorescein-labeled construct **A** with dimer **2** in a binding buffer consisting of 10 mM Na_2PO_4, 1 mM $MgCl_2$, 100 mM NaCl, pH 7.20, the anisotropy value of the Fl-labeled construct **A** was observed to increase and reached saturation when ~10 μM of construct **A** was added (excitation at 490 nm and monitored at the emission wavelength of 520 nm; Fig. 3A). The anisotropy measurements were performed at 15 °C using a Yobin-Horiba Fluorolog-3 (Edison, NJ) and a 0.3 cm pathlength quartz cuvette (300 μl vol). Repeating the same experimental procedures with all the remaining constructs **B** to **K**, it was observed that a slightly higher concentration of constructs **B**, **C** and **D** (~13–18 μM) is required to achieve saturation in their anisotropy values. As for constructs **E**, **F**, **G** and **H**, there was barely any increase in the anisotropy value when the constructs were titrated with increasing concentration of dimer **2** (up to 500 μM). Collectively, these observations indicate that binding interaction does indeed occur between **2** and constructs **A**, **B**, **C** and **D**, but there are no binding against constructs **E**, **F**, **G** and **H**. The extrapolated dissociation constants of **2** against all the nucleic acid constructs is summarized in Table 1, which clearly shows that the binding affinity of dimer **2** is of the following trend: constructs **A** > **B** ≈ **D** > **C**.

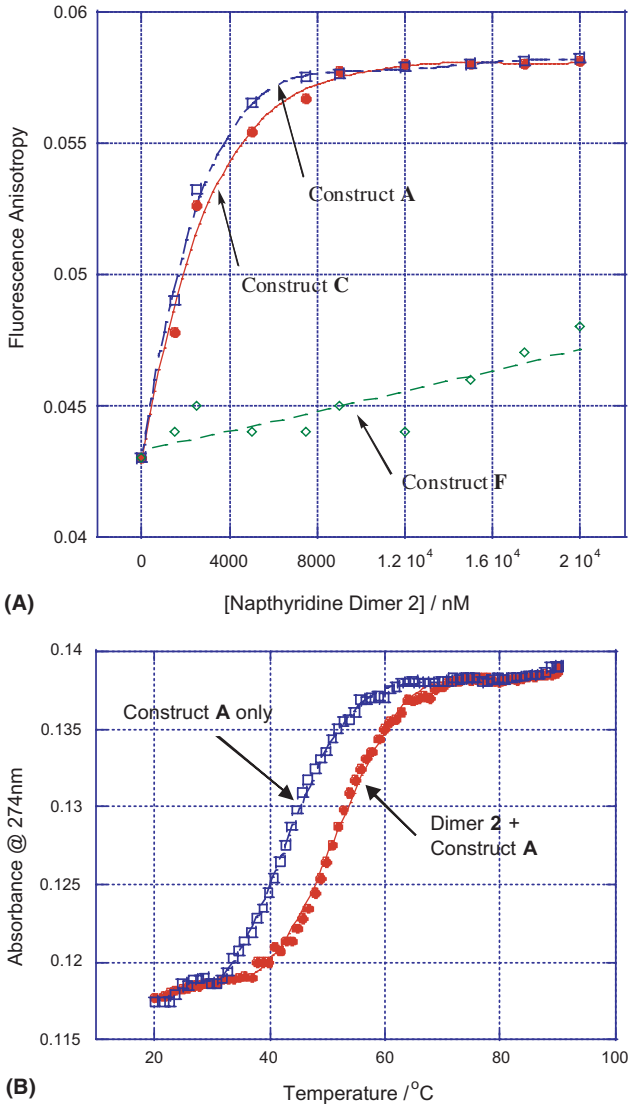

Figure 3. (A) Representative fluorescence titration anisotropy plots of the fluorescein-labeled duplex construct **A**, **C** and **F** (10 nM) as a function of increasing concentration of napthyridine dimer **2**; (B) Representative thermal denaturation plot for construct **A** before and after the addition of 3 equiv of napthyridine dimer **2**.

Table 1. Summary of (i) dissociation constants (K_d) of the nucleic acid constructs **A** to **H** against dimer **2** and nucleic acid constructs **I** to **K** against monomer **1**, and (ii) changes in the melting temperature (ΔT_m) upon addition of either ligands **1** or **2** with the nucleic acid constructs

RNA constructs	Dissociation constants (K_d)/μM	Changes in melting temperature (ΔT_m)/°C
A	1.4 ± 0.09	+9.6
B	2.4 ± 0.14	+8.5
C	2.9 ± 0.19	+8.9
D	3.2 ± 0.24	+7.2
E	N.B.	−0.3
F	N.B.	+0.2
G	N.B.	+0.1
H	N.B.	+0.2
I	35.1 ± 4.0	+1.9
J	32.3 ± 3.9	+1.7
K	34.9 ± 3.4	+1.2

N.B. = no measurable binding.

For monomer **1**, it was observed that **1** was able to also bind constructs **I**, **J** and **K** in a saturable fashion. A much higher concentration of ~500 μM is required for **1** to achieve saturation compared to dimer **2**, indicating that the K_d values are much higher between the interacting species (figure not shown, K_d values summarized in Table 1). However, the binding affinity of **1** is quite similar to constructs **I**, **J** and **K**, indicating that there is no preference for the nature of the nucleic acid targets.

3. UV thermal denaturation measurement

UV thermal denaturation experiment was performed to investigate how the binding of napthyridine ligands affects the melting behavior of constructs **A** to **K**. Denaturation UV plots for the nucleic acid constructs **A** to **K** were obtained using a Perkin-Elmer Lambda 25 equipped with a PTP-1 Peltier control (Norwalk, CT) with its UV measurements taken at 274 nm ([nucleic acid] = ~50 μM; binding buffer = 10 mM Na_2PO_4, 1 mM $MgCl_2$, 100 mM NaCl, pH 7.2) with a gradual temperature increase of 1 °C/min. Through analysis of the obtained denaturation plot with the Perkin-Elmer TempLab software ver. 2.0, the T_m values for constructs **A** to **K** were elucidated. Upon the complexation of

150 μM (3 equiv) of either ligands **1** or **2** to their respective nucleic constructs, that is, constructs **A** to **H** against ligand **2** and constructs **I** to **K** against ligand **1**, the same denaturation experimental approach was repeated. Again through analysis of the UV denaturation plot with the same Templab software, the T_m values for the napthyridine ligand–nucleic acid complexes were obtained. The changes in the melting temperature for each constructs (ΔT_m) were subsequently calculated and the obtained data are summarized in Table 1. First, it is observed that dimer **2** results in a T_m value increase of +7.2 °C to +9.6 °C for the nucleic acid duplexes containing G–G mismatches, whereas there is negligible enhancement in T_m for the four control constructs (**E** to **H**) that do not contain the G–G mismatch (Table 1, with a representative thermal plot for construct **A** before and after addition of 3 equiv of dimer **2** shown in Fig. 3B). These results suggest that **2** is binding specifically to the G–G mismatched bases within constructs **A** to **D**, and is unaffected by the conformations of the nucleic acid target constructs. Second, it was observed that monomer **1** is also able to enhance the T_m values of constructs **G** to **I** by a modest value of +1.2 °C to +1.9 °C. Nevertheless, it indicates that napthyridine monomer is also able to recognize the single G-bulge in both RNA duplex and RNA–DNA heteroduplex constructs.

4. Circular dichroism measurement

To investigate if the titration of ligands **1** and **2** affects the secondary structure of nucleic acid constructs **A** to **I**, circular dichroism (CD) spectra were recorded for all the constructs before and after titration of the ligands. The spectra were recorded at 5 °C with a JASCO J-810 CD spectropolarimeter equipped with a PTC-423S Peltier (Easton, MD) using a 0.1 cm pathlength quartz cuvette (binding buffer = 10 mM Na_2PO_4, 1 mM $MgCl_2$, 100 mM NaCl, pH 7.2). The CD spectra obtained for constructs **B**, **C** and **D** all exhibit a large positive band at ~268 nm, which are indicative of a canonical A-conformation (data not shown). On the contrary, the CD spectrum for construct **A** showed a large positive band ~275 nm maximum, indicative of a canonical B-conformation. Upon addition of 3 equiv of either ligands **1** or **2** to their respective target constructs, the CD spectra were again collected. In general, there are no obvious shifts in the wavelength of the positive band, indicating that the secondary structures of the resulting constructs, upon complexation with the binding ligand, did not undergo any drastic changes. This observation is consistent with the CD spectra obtained for other RNA-small molecule and RNA-peptide interacting systems as previously reported.[29,30]

5. Conclusion

Mismatched bulges in nucleic acid constructs are important in the recognition event between biological molecules. Specifically, numerous small molecular or peptidic ligands, for example, aminoglycoside antibiotics, HIV-1 Tat peptide and HIV-1 Rev peptide are reported to bind RNA targets primarily at 'mismatched' stem loops. The ability to design molecules capable of specific binding to these stem loops would thus generate potential antagonists in impeding the RNA-small molecule or RNA-peptide interacting processes.[7,31–35] Herein, results obtained indicate that napthyridine dimer **1** is able to specifically bind G–G mismatches in nucleic acid constructs comprising of RNA–RNA, RNA–DNA and DNA–DNA duplexes. The observed binding behavior of napthyridine dimer **1** highly suggests that interaction is primarily occurring between the guanine bases and the napthyridine moiety, and is independent of the overall structure of the nucleic acid duplexes. With the observation that the binding characteristics of napthyridine dimer **1** is solely dependent on the identities of the bases that comprise the internal bulge, the current effort is now focused on the adjustment of the recognition module such that other bases could be encompassed. The results of the ability of napthyridine dimer **1** to compete with the HIV-1 Rev peptide-RRE RNA binding process will be reported shortly.

Acknowledgements

This work was supported by the National Institutes of Health (GM #08153-26) and the ACS-PRF grant (#37624-GB4). M.S. is grateful to the McNair Scholars Program and the LSAMP Program for support.

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