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Aminoglycoside Antibiotics, Neamine and Its Derivatives as Potent Inhibitors for the RNA–Protein Interactions Derived from HIV-1 Activators

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Abstract—Neamine derivatives which have an arginine (RN), a pyrene (PCN) and both pyrene and arginine (PRN) have been prepared and their binding toward the RNA fragments derived from HIV-1 activator region, TAR and RRE RNA were examined. Among them, PRN bound either TAR RNA or RRE RNA with equivalent binding affinities as Tat and Rev peptide, respectively.

A design of the molecules that bind RNA fragment is currently an interesting and important issue in drug discovery. However, since RNA can form intricate structure, there is no solid rule to design RNA binding molecules. Combinatorial chemistry is one method of finding a molecule which binds specific target, although it may not be easy to figure out the relationship between specific binding and chemical structure. Here, we would like to report an approach to construct an RNA binding molecule with relatively simple scaffold and building blocks.

The replication of human immunodeficiency virus type 1 (HIV-1) is activated by two RNA–protein interactions.[1–4] One of them is the transactivator protein (Tat) and its responsive RNA element (TAR), and the other is the regulator of virion expression (Rev) and its responsive RNA (RRE). Obviously, electrostatic interaction is important for the binding with RNA fragment, because RNA itself is a negatively charged phosphate-pentose polymer, though, for high specificity of the molecular recognition toward RNA target, an organized structural feature should be required. While Tat peptide has no solid structure, Rev peptide forms α-helix upon binding with the RNA. Alternatively, some structurally related aminoglycoside antibiotics, such as neomycine B, paromomycin, tobramycin, and kananycin, bind RNA specifically and disturb their activity.[5–9] For elucidation of the rule for the molecular recognition performed by RNA, aminoglycoside–RNA complex must be a nice model.[10–12] Recent studies suggest that neomycine binds either TAR or RRE RNA specifically and disturbs corresponding RNA–protein interactions. Even their binding site is not exactly the same as that of the protein, aminoglycosides inhibit RNA–protein interaction in a non-competitive way.[13–15] Under these circumstances, the binding affinities of several structurally related aminoglycosides were determined by fluorescent binding assay which we have recently developed, and neamine was known as a consensus unit of aminoglycosides which bind either RRE IIB RNA or TAR RNA.[16,17] Neamine has been chosen as a scaffold for the construction of the molecules that bind specific RNA fragments. The RNA binding regions of both Tat and Rev proteins are known as arginine (R) rich peptide sequences. It is also known that these small peptides have almost the same affinity as whole protein for the corresponding RNA. $Tat_{49–57}$ and $Rev_{34–50}$ include six and 10 arginines, respectively (Fig. 1). The conjugation of neamine with arginine might enhance binding affinity of the compound toward the RNA. Also, several aromatic compounds, such as pyrene, are known as intercalator or as stacker for double stranded polynucleotides.[18] On this basis, substituted neamines (Fig. 2) bearing an arginine (RN) or a pyrene (PCN) and both pyrene and arginine (PRN) have been prepared. PRN was synthesized

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by the condensation reaction of neamine and amino group protected arginine, Fmoc-Arg(Mtr)OH, followed by removal of the Fmoc-protection group by piperidine. α-Amino group of the arginine was labeled with pyrenecarbonyl, using corresponding succiimidyl ester (PyOSu). Then 4-methoxy-2,3,6-trimethylbenzenesulfonyl (Mtr) group was removed by trimethylsilyl bromide (TMSBr) in trifluoroacetic acid (TFA). PCN was synthesized as previously reported.[16] The products were purified by reversed-phase HPLC, and then characterized by ^{1}H NMR[19] and MALDI-TOF MS.[20] The concentrations of neamine and its derivatives that perform 50% inhibition (IC_{50}) for either TAR–Tat or RRE–Rev complex were determined by the competitive binding assay using corresponding fluorescent peptide (Fig. 1) as a tracer.[16,17] Alternatively, due to relatively strong emission of the pyrene moiety, the dissociation constants (K_D) of PCN and PRN were determined directly from the decrease in the fluorescence of the pyrene moiety upon the addition of the RNA.

The IC_{50} of neamine for TAR and RRE RNA were determined as 28 and 21 μM, respectively (Table 1). The binding affinities are 1000 and 7000 times weaker than that of the corresponding peptides. Since arginine is the main component of either Tat or Rev peptide, it is expected that arginine substitution may enhance the binding affinity of neamine toward the RNA. As expected, arginine substituted neamine (RN) inhibited Tat and Rev peptide binding toward the RNA with the IC_{50} values of 600 and 460 nM, respectively (Table 1). Single arginine substitution on neamine results in 47-fold enhancement of inhibition for RNA–protein binding compared with that of neamine itself. These results suggest that an arginine moiety in RN enhances the binding for the RNA by forming another hydrogen bond. On the other hand, appropriate substitution of aromatic compound also enhances the binding affinities of aminoglycosides. Pyrenecarbonyl labeled neamine (PCN) was prepared, and its binding with either TAR or RRE RNA was examined. IC_{50} values of PCN for TAR and RRE RNA were 1700 and 19,000 nM, respectively (Table 1). While pyrene-substitution enhanced the inhibitory effect for TAR–Tat 16 times higher than that of neamine, it did not enhance the inhibitory effect for RRE–Rev complex. On the other hand, when the solution of PCN (100 nM) was titrated by the RNA directly, pyrene fluorescence of PCN decreased with increasing concentration of the RNA. Then K_D values were determined as 290 and 240 nM for TAR and RRE, respectively (Table 2). Although there is a large difference between IC_{50} and K_D of PCN, it was shown that pyrene substitution has potential to enhance the aminoglycoside binding toward the RNA. According to recent binding analysis using gel shift assay, RRE RNA has more than two binding sites for neomycin and it was

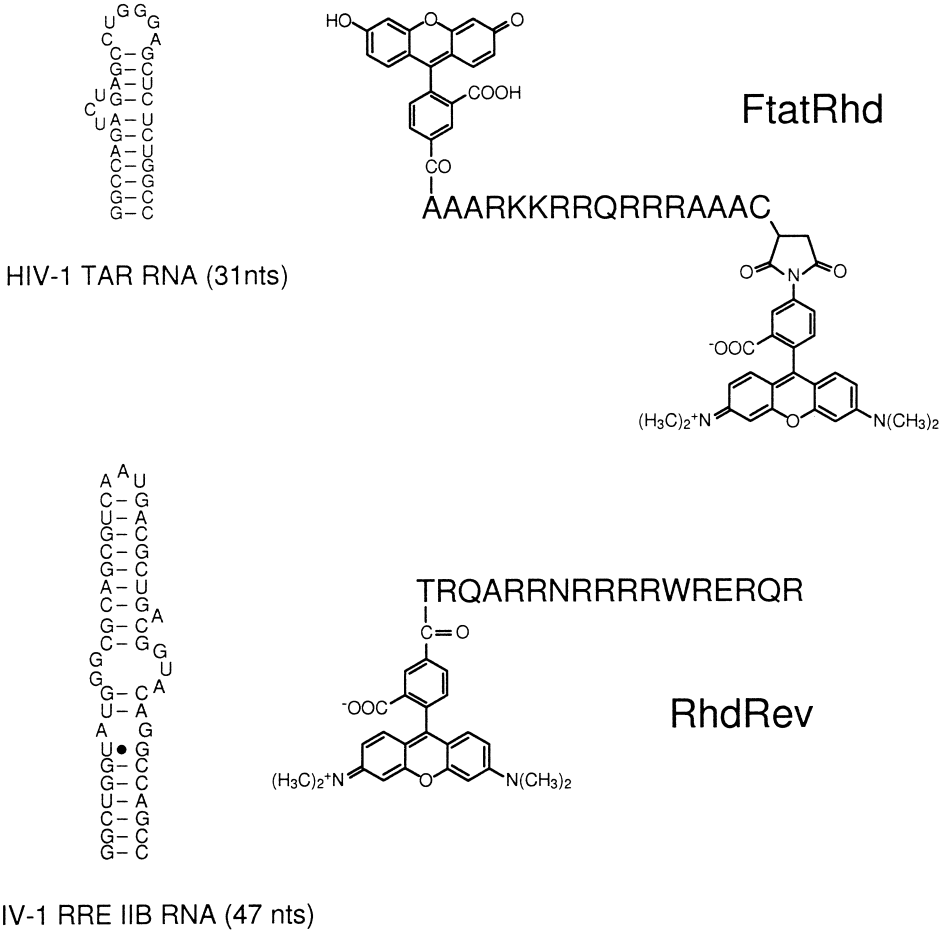

Figure 1. Secondary structure of TAR and RRE RNA, and dye tagged Tat and Rev peptides.

shown that the first binding site has a higher affinity than the second one.[15] Since neomycine includes neamine unit as RNA binding core, RRE RNA may bind more than two neamine derivatives. Direct binding analysis by the pyrene fluorescence of PCN might represent the binding affinities of the first binding site. For the sake of developing binding specificity of PCN, an arginine was introduced as a linker between neamine and the pyrene (pyrene–arginine–neamine conjugate, PRN). PRN inhibited each peptide binding to TAR and RRE RNA with the IC_{50} values of 28 and 350 nM, respectively (Table 1). These inhibitions are 1000 and 60 times higher than that of neamine itself. Also, K_D of PRN with the RNAs were determined by the fluorescence change of PRN as a function of the RNA concentration and analyzed with the equation of 1:1 stoichiometry. The obtained data points were fit with theoretical curves finely (Fig. 3). These results indicated that the bindings of PRN were stoichiometric and the analysis gave 30 and 8.5 nM as dissociation constants for TAR and RRE RNA, respectively (Table 2). Those binding affinities are the same level of magnitude as that of the regulator

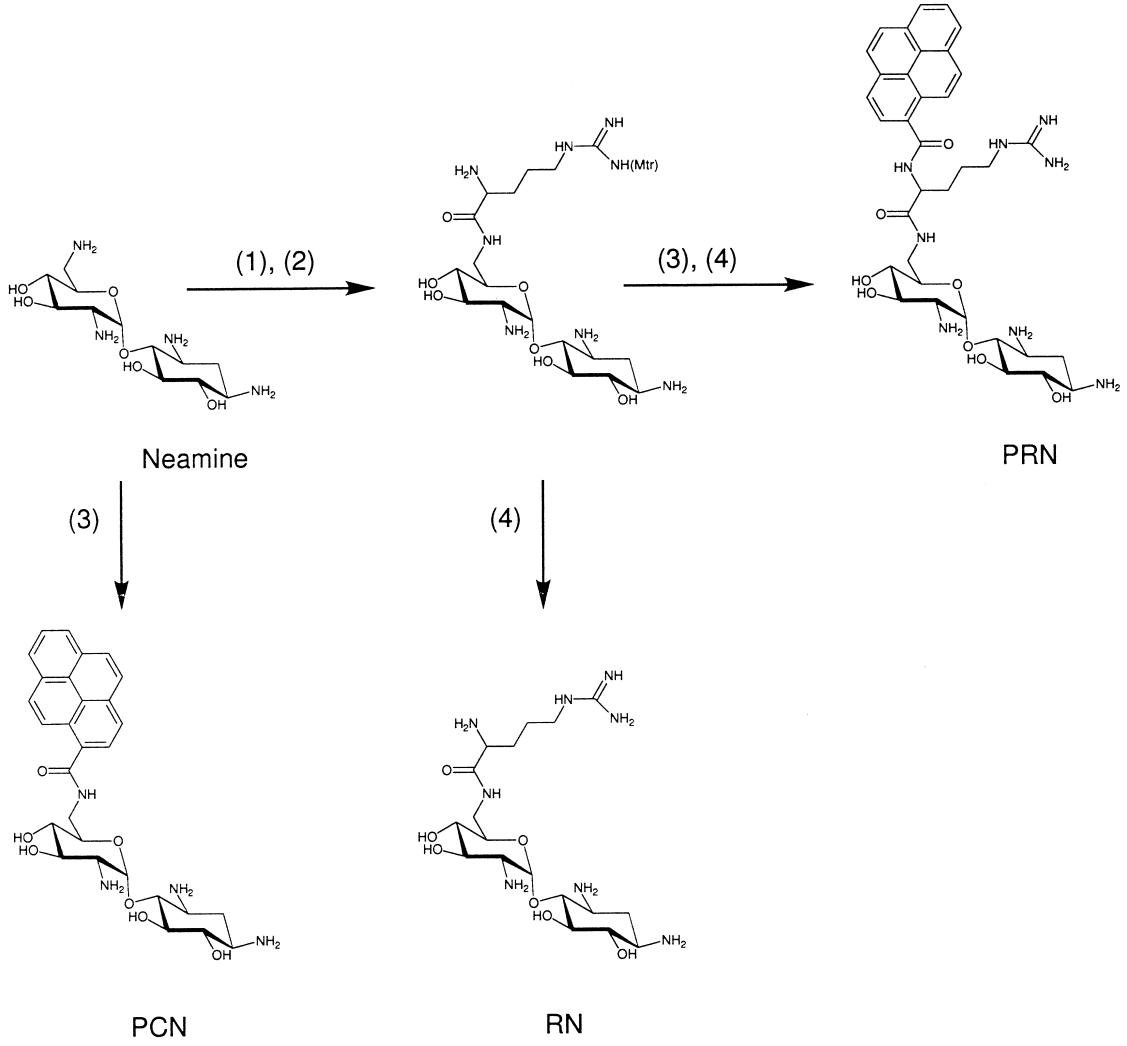

Figure 2. Synthesis of RN, PCN and PRN. Reagents and conditions: (1) Fmoc-Arg(Mtr)OH, WSC, DMF, water, room temperature, 16 h; (2) Piperidine, DMF; (3) PyOSu, DMF, water, room temperature, 20 h; (4) TMSBr, TFA, 0 °C, 2 h.

Table 1. IC_{50} (nM) of neamine and its derivatives for HIV-1 TAR and RRE IIB RNA

	Neamine	RN	PCN	PRN
TAR	28,000	600	1700	28
RRE	21,000	460	19,000	350

Table 2. Dissociation constants (nM) of neamine and its derivatives for HIV-1 TAR and RRE IIB RNA

	Peptide	PCN	PRN
TAR	25[a]	290	30
RRE	3.3[b]	240	8.5

[a]$Tat_{49–57}$ peptide.
[b]$Rev_{34–50}$ peptide.

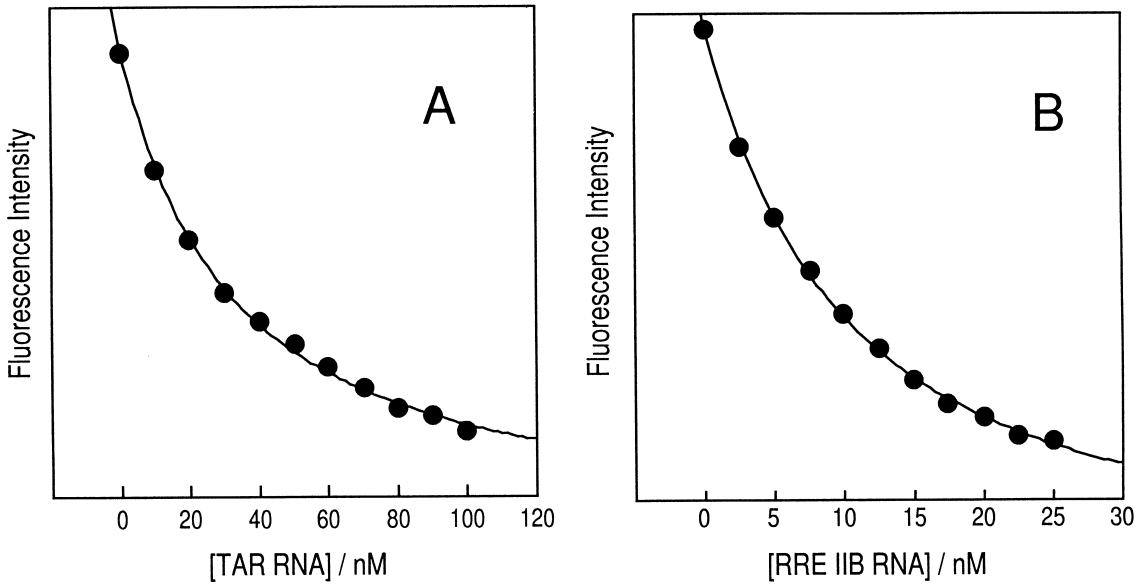

Figure 3. Fluorescence intensity at 386 nm of PRN, as a function of (A) TAR RNA and (B) RRE RNA concentrations. Assay conditions: 20 mM Tris–HCl buffer containing 140 mM NaCl, 5 mM $MgCl_2$, pH 7.5 at 25 °C, excitation at 340 nm.

proteins, Tat and Rev. The IC_{50} and the K_D of PRN for TAR RNA are almost the same as that of Tat. Recent study of the structure of TAR–neomycin complex revealed that the binding site of TAR RNA for Tat and that of neomycine is not exactly the same.[14] However the results shown above suggest that the inhibition of PRN for TAR-Tat is considerably competitive. PRN binding may reduce the Tat-accessible surface in TAR RNA in a similar way as that of neomycine binding.[14] K_D of PRN for RRE RNA was also same level as that of Rev. Although there is a large difference between IC_{50} and K_D for RRE RNA, the binding affinity of PRN for RRE RNA was surprisingly enhanced. But, unfortunately, PRN did not disturb RRE–Rev interaction as efficiently as that for TAR–Tat. Since RRE RNA has a distinct binding site for neomycin and Rev, RRE RNA may have an independent binding site for PRN.

As expected, the conjugation of aminoglycoside antibiotic, neamine as a scaffold, arginine as a basic amino acid and aromatic ring, pyrene as a stacker successfully enhanced the binding affinity of neamine toward the RNA. PRN is the molecule having multiple intermolecular interactions including electrostatic, hydrogen bonding and aromatic stacking with the target RNA. These results demonstrate that the molecule having multiple binding interaction is capable of binding the target RNA as strongly as that of the protein, even if it is a relatively simple and small molecule.

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19. ^1H NMR (in D_2O, 300 MHz), RN 1.13 (m, 2H), 1.54 (m, 4H), 1.87 (m, 2H), 2.65–3.71 (m, majority), 5.02 (q, 1H), 5.22 (d, 1H), PRN 1.19 (m, 7H), 1.23 (q, 1H), 2.97–3.90 (m, majority), 5.70 (d, 1H), 5.57 (d, 1H), 8.19 (m, 9H).
20. MALDI-TOF MS, found $M+Na^+$ (calcd $M+Na^+$), RN 493.6 (492.6), PRN 728.9 (728.8)