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An aminoglycoside antibiotic, neamine, and its aromatic ring-substituted derivatives as potential inhibitors for HIV-1 RRE-Rev

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Abstract

The binding affinities of ten aminoglycosides with RRE IIB RNA have been determined. Neamine was found as the simplest consensus unit of aminoglycoside antibiotics that bind RRE IIB RNA tightly. Various aromatic ring-substituted neamines have been prepared and their binding with RRE IIB RNA was examined. Among them, pyrene-substituted neamine has shown a submicromolar level of binding with the RNA. © 2000 Elsevier Science Ltd. All rights reserved.

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In the replication of humanimmunodeficiency virus type 1 (HIV-1), two pairs of protein and the corresponding RNA play a critical role. One of these is a trans activator protein (Tat) and its responsive mRNA fragment, trans activator responsive element (TAR), and the other is a retro viral protein (Rev) and its responsive mRNA, Rev responsive element (RRE).[1–3] A molecule which interrupts those protein–RNA interactions must work as an antagonist to the replication of HIV. Also, the discovery of those molecules should be helpful towards understanding the molecular recognition of RNA. Several aminoglycosides are known to bind either TAR RNA or RRE RNA and disturb the RNA–protein binding.[4,5] RNA–aminoglycoside interaction could be a nice model for the elucidation of the molecular recognition performed by RNA.[6,7] On the other hand, anthracene, acridine or similar aromatic compounds are known as stackers for either double-stranded RNA or DNA.[8] The combination of an aminoglycoside and an aromatic ring might enhance both the affinity and specificity of the molecule.

The binding site of HIV-1 RRE RNA and TAR RNA is a relatively small fragment composed of 47 and 31 nucleotides, respectively. (RRE IIB RNA, Fig. 1.) Also, the binding domains of the

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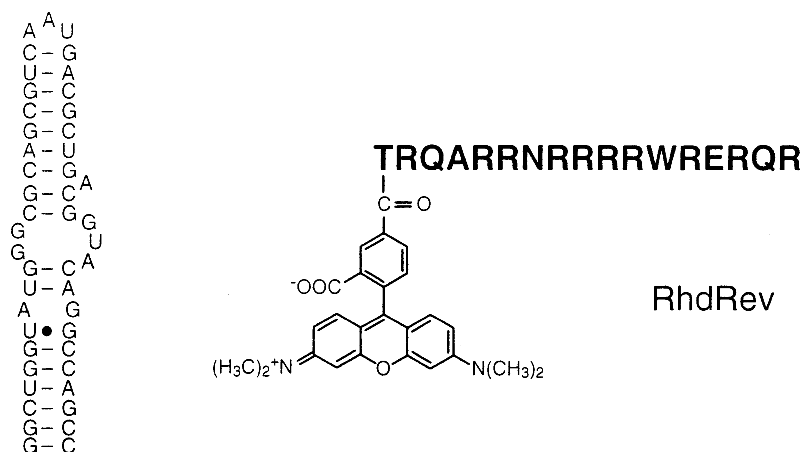
Rev and Tat proteins have been characterized extensively, and it is known that the binding domains of the Rev and Tat proteins are small fragments of the peptide composed of 17 and 9 amino acids and called Rev peptide ($Rev_{34–50}$) and Tat peptide ($Tat_{49–57}$), respectively. It is also known that the binding affinity of Rev and Tat peptides with the RNA is equivalent to that of a corresponding whole protein.[9,10] Recently, we have developed and evaluated a binding assay utilizing fluorescent resonance energy transfer (FRET) with the intrinsically flexible $Tat_{49–57}$ peptide. Upon binding with TAR RNA, dynamic structural change of Tat peptide results in dramatic fluorescence change.[11] On the other hand, the Rev peptide is known to induce an α-helix structure in the complex of RRE RNA.[12–14]



HIV RRE IIB RNA (47 nts)

Figure 1. Secondary structure of RRE IIB RNA and tetramethylrhodamine labeled Rev peptide

On this basis, tetramethylrhodamine labeled Rev_{34-50} peptide was prepared (RhdRev, Fig. 1) as a tracer. When Rev peptide forms a complex with the RNA, a drastic change in the mobility of Rev peptide is expected, which causes a fluorescence anisotropy increase of RhdRev. On the other hand, when the antagonist competes with RhdRev and binds with the RNA, the binding of the antagonist is observed as a decrease of fluorescence anisotropy. $Rev_{34–50}$ peptide was synthesized by the stepwise elongation of Fmoc-protected amino acids and labeled with the dye on the resin, then purified by HPLC and identified by MALDI-TOFMS.[15] The dissociation constants of wild-type Rev peptide and RRE IIB RNA were successfully determined as 3.3 nM by the competitive binding assay[11] utilizing fluorescence anisotropy of RhdRev. Since aminoglycoside works as an antagonist for the replication of HIV-1, it is of interest to determine accurate dissociation constants of those aminoglycosides. The structural relationship of the RNA binding aminoglycosides should be elucidated, and one of them might be chosen as a scaffold for the development of potential inhibitors against HIV-1 replication. Ten aminoglycosides (Fig. 2) were chosen, and their dissociation constants with RRE IIB RNA have been determined by a binding assay utilizing fluorescence anisotropy of RhdRev as the tracer. These results are summarized in Table 1. Neomycin has the highest binding affinity (4.4 μM) with RRE IIB RNA. Binding affinities of all other aminoglycosides were weaker (over 10 μM) than that of neomycin. The dissociation constants of hygromycin B and streptomycin were determined as 248 and 200 μM, respectively. Hygromycin B was known as the weakest binder among the

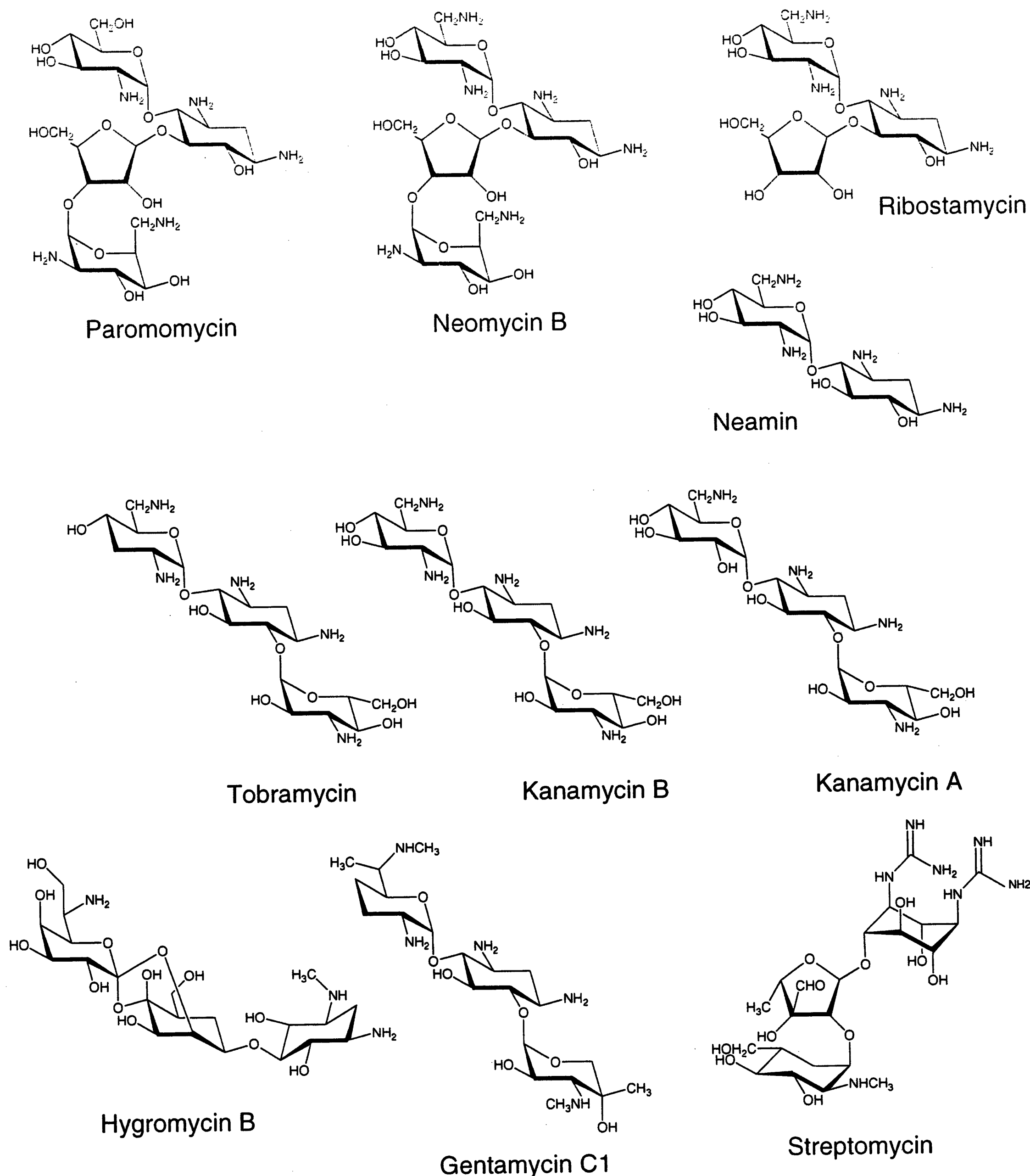


Figure 2. Aminoglycosides with which their binding toward HIV-1 RRE IIB RNA was examined

aminoglycosides examined here. Interestingly, the chemical structures of those aminoglycosides which bind RRE IIB RNA are close to each other, and it is easily found that neamine is the smallest and the simplest aminoglycoside that has a consensus unit of those aminoglycosides. On the other hand, the compounds including an aromatic ring are capable of intercalating with the major groove or stack with the base of polynucleotides. Under these circumstances, neamine was chosen as a scaffold for the RNA binding site, and a series of aromatic substituted neamines

Table 1
Dissociation constants of aminoglycosides for RRE IIB RNA[a]

Aminoglycosides	K_D (μM)
Gentamycin	34.6 ± 6.1
Hygromycin B	248 ± 19
Kanamycin A	46.0 ± 3.5
Kanamycin B	14.4 ± 1.4
Neomycin	4.38 ± 0.48
Neamin	20.8 ± 3.4
Paromomycin	34.5 ± 2.7
Ribostamycin	87.5 ± 6.3
Streptomycin	200 ± 83
Tobramycin	15.5 ± 5.6

[a] Assay condition: 20 mM tris–HCl buffer containing 140 mM NaCl, 5 mM KCl, 1 mM MgCl, pH 7.5, at 25°C.

were prepared (Fig. 3) and their binding affinities with RRE IIB RNA were examined. All the aromatic substituted neamines were prepared by a condensation reaction of neamine and the corresponding aromatic carboxylic acid succinimidyl ester, then purified by reversed-phase HPLC and characterized by ^{1}H-NMR[16] and MALDI-TOFMS.[17]

The binding affinities of benzoyl, 2-naphthoyl and anthracene-9-carbonyl substituted neamine derivatives, BCN, NCN and CAN, respectively, were determined by the competitive binding assay using RhdRev as a tracer. On the other hand, due to the relatively strong fluorescence emission of the pyrene unit, binding affinities of pyrene-substituted neamines were determined directly from the fluorescence of the pyrene that conjugates with neamine. The binding results are summarized in Table 2. The substitution on the amino group with either a benzoyl or a 2-naphthoyl moiety disturbs the electrostatic interaction, then weakens the binding affinities of those aromatic-substituted neamines. Benzene and naphthalene must be too small to stack with polynucleotides, and substitution on the primary amino group of neamine weakened the binding. In contrast, the anthracene-9-carbonyl moiety-substituted neamine has six times higher affinity with the RNA than native neamine. Anthracene is the simplest unit that is capable of intercalating with double-stranded polynucleotides. Namely, intercalation of the anthracene unit enhanced the binding of the modified neamine with the RNA. Fig. 4 shows the fluorescence spectra of pyrene carbonyl substituted neamine (PCN) alone and in the presence of the RNA (A) and its intensity plots at 385 nm as a function of the RNA concentration (B). The solid curve was obtained by the equation of 1:1 stoichiometry.[11] All the experimental data were fit to the solid curve finely and gave 0.24 μM as the dissociation constant. This result suggests that PCN binds RRE IIB RNA with 1:1 stoichiometry and remarkably enhanced its binding affinity with the RNA. As with PCN, the dissociation constants of other pyrene-substituted neamines, pyreneacetyl-substituted neamine (PAN), and pyrenebutanoyl-substituted neamine (PBN) were determined as 0.75 and 0.28 μM, respectively. The binding affinities of these pyrene-substituted neamines, PCN, PAN and PBN are 87, 28 and 75 times higher than that of native neamine, respectively. These results suggest that the pyrene unit may intercalate with the groove or stack with the base in the RNA and enhance the binding affinity of the pyrene-substituted neamines. According to the binding results of pyrene-substituted neamines, the length of the linker which connects the pyrene unit and neamine has little effect on the binding affinity. All these results

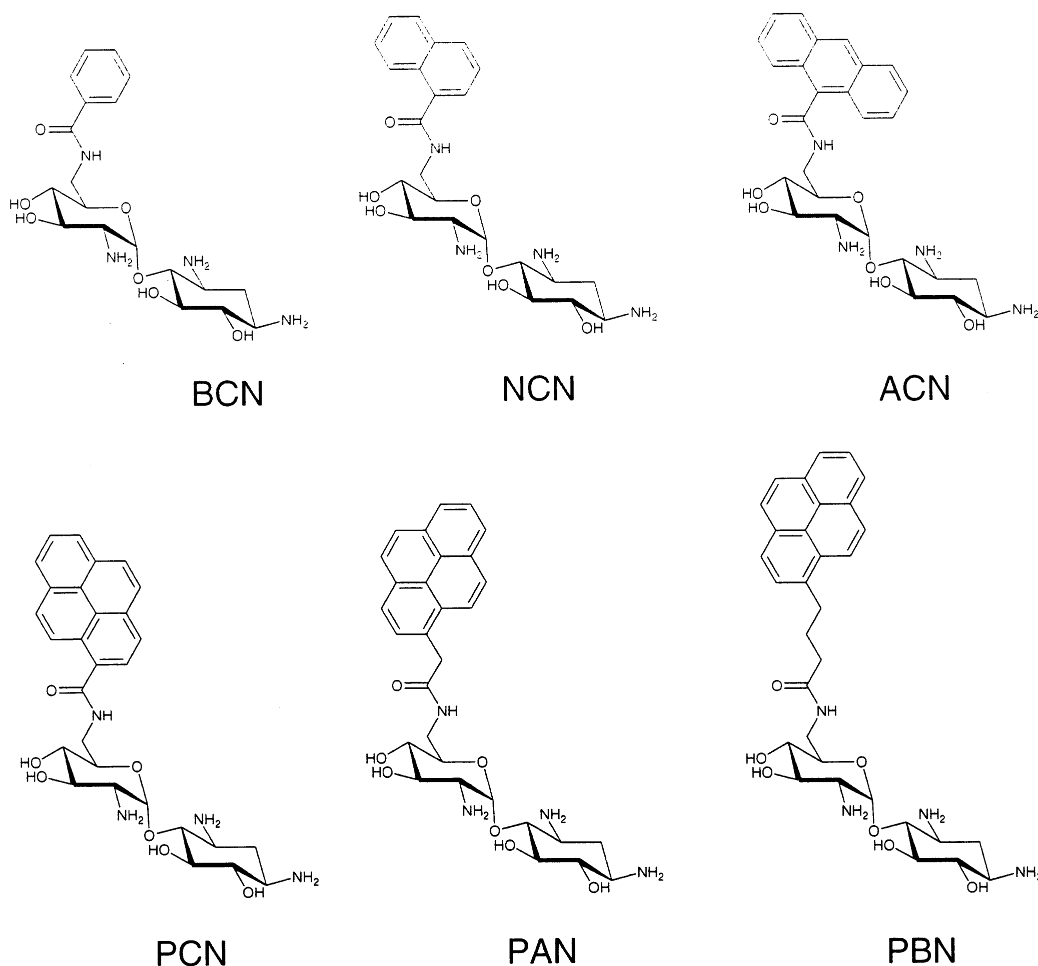


Figure 3. Aromatic ring substituted neamines

Table 2
Dissociation constants of the aromatic ring substituted neamines for RRE IIB RNA[a]

Inhibitors	K_D (μM)
Neamine	20.8 ± 3.4
BCN	141 ± 7
NCN	40.6 ± 2.2
ACN	3.6 ± 0.7
PCN	0.240 ± 0.060
PAN	0.753 ± 0.045
PBN	0.277 ± 0.014

[a] Assay condition: 20 mM tris–HCl buffer containing 140 mM NaCl, 5 mM KCl, 1 mM MgCl, pH 7.5, at 25°C.

shown above suggest that neamine should be a good scaffold to construct a novel molecule which binds the RNA fragment specifically. Further study along this line is now under way.

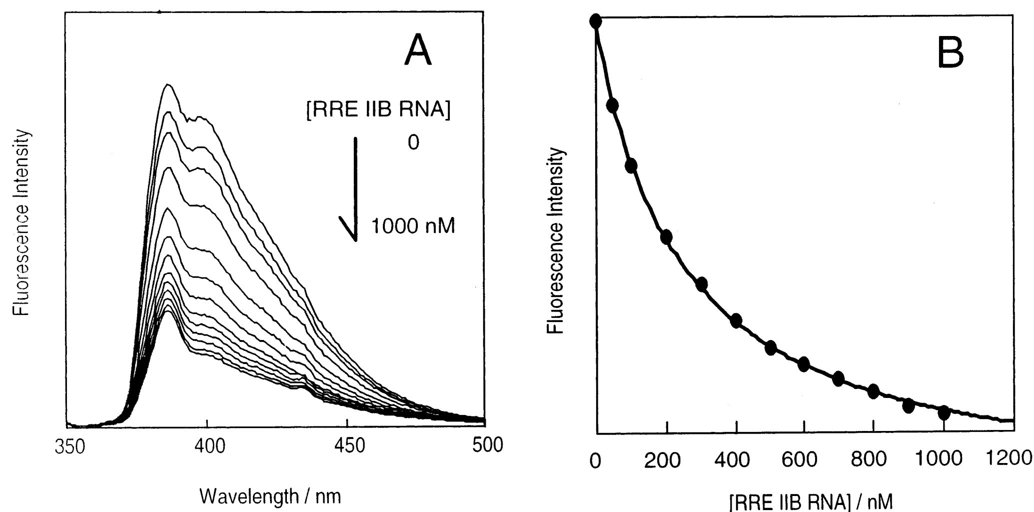


Figure 4. (A) Fluorescence spectra of PCN in 20 mM tris–HCl buffer containing 140 mM NaCl, 5 mM KCl, and 1 mM $MgCl_2$ pH 7.5 at 25°C, excitation wavelength: 340 nm. (B) Titration curve of PCN with HIV-1 RRE IIB RNA, the curve was obtained by the curve-fitting analysis based on the equation of 1:1 stoichiometry

Acknowledgements

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15. MALDI-TOFMS, found $M+H^+$ (calcd $M+H^+$), Rev 2538.4 (2537.9), RhdRev 2883.0 (2882.8).
16. ^{1}H-NMR (300 MHz, D_2O), BCN 1.68 (q, 1H), 2.33 (m, 1H), 3.01–3.83 (m, majority), 5.57 (d, 2H), 7.38 (t, 2H), 7.48 (t, 1H), 7.62 (d, 2H); NCN 1.68 (q, 1H), 2.35 (m, 1H), 3.13–3.955 (m, 7H), 3.70–3.90 (m, 6H), 5.64 (d, 1H), 7.48 (m, 4H), 7.92 (m, 3H); ACN 1.70 (q, 1H), 2.29 (m, 1H), 2.64 (m, 4H), 2.97 (br, 1H), 3.21–3.75 (m, majority), 5.50 (d, 1H), 7.49 (t, 2H), 7.57 (t, 2H), 8.98 (d, 4H), 8.81 (s, 1H); PCN 1.78 (q, 1H), 2.42 (m, 1H), 3.24–3.64 (m, 8H), 3.86–4.02 (m, 6H), 5.78 (d, 1H), 8.07 (m, 9H); PAN 1.62 (q, 1H), 2.32 (m, 1H), 2.62 (m, 1H), 3.14 (m, 1H), 3.26–3.68 (m, 9H), 5.23 (d, 1H), 8.01 (m, 10H), PBN 1.67 (q, 1H), 1.86 (t, 2H), 2.18 (t, 2H), 2.32 (m, 1H), 2.91–3.70 (m, 15H), 5.42 (d, 1H), 7.73 (m, 9H).
17. MALDI-TOFMS, found $M+H^+$ (calcd $M+H^+$), BCN 426.4 (426.5), NCN 476.9 (476.5), ACN 526.3 (526.6), PCN 558.9 (560.4), PAN 566.0 (564.4), PBN 587.7 (588.4).