A Peptide Nucleic Acid−Neamine Conjugate That Targets and Cleaves HIV-1 TAR RNA Inhibits Viral Replication†

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Abstract: The neamine part of the aminoglycoside antibiotic neomycin B was conjugated to a 16 mer peptide nucleic acid (PNA) targeting HIV-1 TAR RNA. Attachment of the neamine core allows cellular uptake of the PNA and results in potent inhibition of HIV-1 replication. The polycationic neamine moiety imparts greater solubility to the PNA and also confers a unique RNA cleavage property to the conjugate which is specific to its target site and functional at physiological concentrations of Mg^{2+}. These properties suggest a potential therapeutic application for this class of compounds.

Antisense strategies employing oligonucleotides have been widely used to inhibit expression of many cellular and viral genes. The efficiency of these molecules has been improved by introducing chemical modifications that increase their cellular uptake, affinity and resistance to cellular nucleases.[1−4] Peptide nucleic acids (PNAs) are a new class of antisense DNA analogues first synthesized by Nielsen and colleagues in 1991.[5] The PNA molecules, devoid of sugar phosphate backbone, have been shown to display high affinity for complementary sequences on RNA and DNA both in single and double stranded forms.[5−7] Initial expectations held that PNAs would quickly enter the field of antisense as gene-specific, nontoxic, and nonimmunogenic agents. However, problems associated with solubility and poor cellular uptake of this class of compounds hampered developments in this direction.[8]

The synthesis of modified PNAs or PNA conjugates presents new means of improving their solubility and cellular uptake.[8] Recently, it has been shown that PNAs conjugated to diethylenetriamine and PNA−neocuproine Zn(II) derivatives acquired a novel property of catalyzing chemical cleavage of their RNA targets in vitro.[9,10] Some reports have described in vivo studies using microinjection[11] and, more recently, carrier peptides[12] or guanidine-based PNA[13] for their efficient biodelivery into the cells. Recently, a membrane-permeating peptide conjugated to a 16 mer PNA **1** (Figure 1) targeting the transactivation response element (TAR) of HIV-1 has been shown to inhibit viral production by infected cells when supplemented in the cell culture medium.[14]

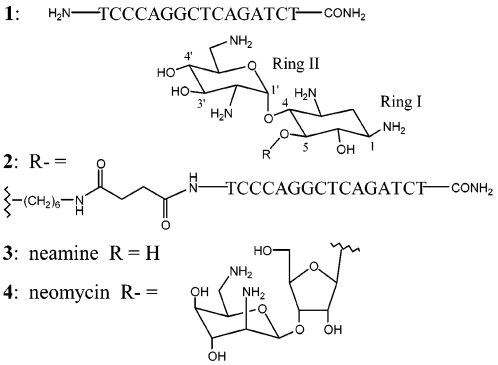

Figure 1. Structure of the anti-TAR PNA **1**, its neamine conjugate **2** and of the aminoglycosides neamine **3** and neomycin **4**.

Here, we report for the first time the synthesis and antiviral efficacy of a PNA−neamine conjugate **2** wherein the neamine moiety **3** not only allows cellular uptake of the PNA **1** targeted to the TAR region of the HIV-1 genome but also confers a RNA-cleaving activity that may enhance its utility as an antiviral agent. Since the TAR element in the LTR is essential for transactivation of HIV-1 transcription and conserved in all HIV-1 isolates, targeting this invariant region may be an interesting strategy for developing new antiviral agents against drug resistant HIV variants.[3,14−16]

Neamine **3** is a constituent of aminoglycoside antibiotics such as neomycin B **4** that are hydrophilic pseudo-oligosaccharides possessing several amino functions that are mostly protonated under physiological conditions. Due to their polycationic nature, they show binding affinity for nucleic acids and bind specifically to 16S bacterial ribosomal RNA (rRNA) and perturb protein synthesis.[17] Neomycin has also been shown to bind the Rev Response Element (RRE)[18] and TAR[19] region in HIV-1 and blocks in vitro interaction of these regions with transacting HIV-1 proteins, Rev and Tat, respectively. However, neomycin B is toxic and prone to enzymatic modifications[20−22] leading to development of high levels of antibiotic resistance. Detailed experiments have shown that rings I and II of the neomycin-class aminoglycosides, corresponding to the neamine structure **3**, are essential structural elements involved in the specific binding to rRNA,[23] RRE[24] and TAR RNA.[25] New chemistries have been developed to synthesize small antibiotics or antiviral agents from neamine.[22,26−30] The high affinity of aminoglycosides for DNA was recently harnessed successfully for transfection of oligo-2′-deoxyribonucleotides (ODN) into cells for gene therapy,[31] and the synthesis of ODN−aminoglycoside conjugates has been recently reported.[32] Aminoglycosides are also able to stabilize nucleic acid triple helices in vitro[33] and aminoglycoside−copper (II) complexes are efficient artificial nucleases that hydrolyze RNA[34] or DNA.[35]

To link the neamine core to PNA sequences at the 5-position corresponding to the attachment position of

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† This letter is dedicated in memory of Professor Claude Hélène.

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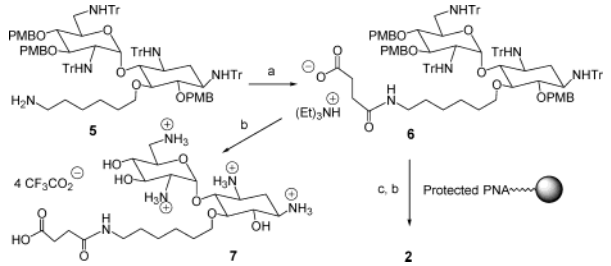
Scheme 1[a] Synthesis and Deprotection of the Neamine Derivative **6** Which Was Conjugated to the Protected PNA **1**



[a] (a) Succinic anhydride, Et_3N, CH_2Cl_2; (b) TFA/anisole (1/1); (c) EDC, HOBt, DMF, protected PNA.

the ribose moiety in neomycin, acid-labile protecting groups, trityl and 4-methoxybenzyl groups, were chosen to protect the amino and hydroxyl functions, respectively. These groups can be easily removed under the conditions used to remove the benzhydryloxycarbonyl (Bhoc) groups employed in the PNA synthesis to protect the amino functions of the bases. The protected neamine derivative **6** (Scheme 1), possessing a carboxylate group necessary for coupling the neamine core to the terminal amino function of the protected PNA, was synthesized in six steps: (1) protection of the four amino groups by reaction of neamine **3** with trityl chloride in triethylamine (77% yield), (2) protection of the 3′, 4′ and 6-hydroxyl functions with 4-methoxybenzyl groups (47% yield, the 5-hydroxyl function is the most hindered hydroxyl function and thus the less reactive), (3) introduction at the 5-position of a hexyl linking chain possessing a terminal bromine atom by reaction of the 5-hydroxyl function with 1,6-dibromohexane in the presence of NaH (76% yield), (4 and 5) substitution of the terminal bromine atom for an azido group (98% yield) and then reduction to obtain, at the end of the hexyl linking chain, an amino function (compound **5**,[36] 60% yield) and (6) reaction of this amino function with succinic anhydride for obtaining **6** (90% yield). For characterization, the neamine derivative **6** was deprotected in TFA/anisole (1:1) to lead to compound **7** in 81% yield (Scheme 1). The derivative **6** was coupled to the protected 16 mer anti-TAR PNA **1** attached to its solid support of synthesis using EDC/HOBt as coupling agents. Deprotection of the neamine core and the PNA bases and cleavage from the solid support were performed in one step in TFA/anisole (1:1). The resulting conjugate **2** was purified by HPLC on C_{18} reversed-phase (50% coupling yield) and its structure was confirmed by mass spectrometry (MS (MALDI): calcd 4792.4768, found 4792.4761).

To determine the ability of the conjugate **2** to bind TAR RNA, we performed gel mobility shift assays using ^{32}P-labeled TAR RNA transcribed from the HIV-1 LTR.[14−16] As seen in Figure 2, a distinct shift in the mobility of TAR RNA was observed due to the formation of a specific [PNA−TAR RNA] complex. This mobility shift was stoichiometric, as a complete shift was observed at 1:1 molar ratio of conjugate to TAR RNA. Binding of neamine alone with TAR RNA could not be detected under our experimental conditions.

Since PNA−RNA or PNA−DNA duplexes exhibit higher T_m than the corresponding RNA−DNA or DNA−DNA duplexes, we wanted to examine whether the

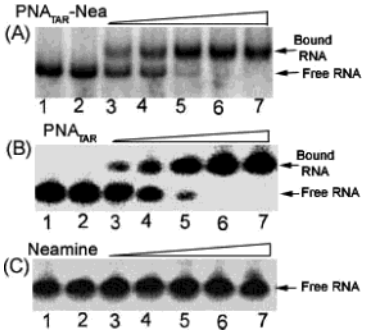


Figure 2. Binding of conjugate **2** to its target sequence on the TAR HIV-1 genomic RNA assessed by gel-mobility shift analysis[16] (Panel A). Unconjugated PNA **1** (Panel B) was included as a positive control and neamine **3** (Panel C) as a negative control. In each panel, lanes 1 through 7 represent molar ratios of ligand to TAR RNA of 0.0, 0.2, 0.5, 0.8, 1.0, 3.0 and 5, respectively.

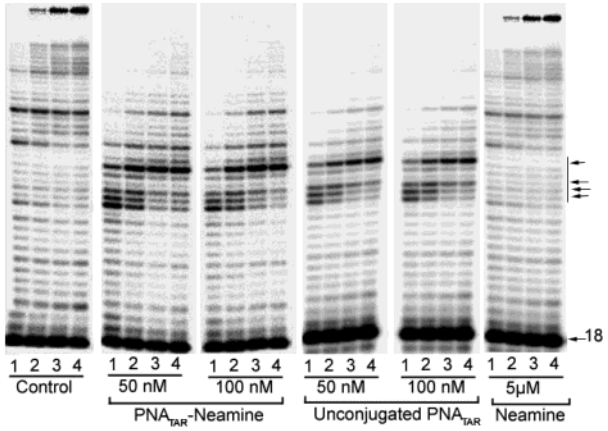


Figure 3. Inhibition of reverse transcription of TAR RNA by conjugate **2**. The TAR RNA template primed with the 5′-^{32}P labeled 18-mer DNA primer was incubated at 25 °C in the absence or presence of increasing concentrations of **2**. Reverse transcription was initiated by the addition of the dNTP mix and HIV-1 RT as described before.[14] PNA **1** and neamine **3** were also used as controls. Lanes 1−4 in each set represent the reaction time for 0.5, 1, 3 and 5 min, respectively. The positions marked with the arrows corresponds to the loop region on TAR RNA targeted by the conjugate **2**.

conjugate **2** was able to block reverse transcription of HIV-1 TAR RNA. Blocking reverse transcription would have multiple effects on viral replication besides influencing Tat-mediated transactivation. For this purpose, TAR RNA primed with the labeled 18-mer DNA primer was incubated in the absence or presence of **2** at 25 °C followed by initiation of reverse transcription by HIV-1 RT. The results presented in Figure 3 illustrate prominent pauses in reverse transcription at the site targeted by conjugate **2**. Similar pauses were also obtained with the unconjugated anti-TAR PNA suggesting that the neamine moiety does not alter the target efficacy of the PNA. These results show that conjugate **2** binds to its target site on TAR and blocks reverse transcription, probably by inhibiting the strand displacement activity of HIV-1 RT. Inhibition of reverse transcription by the conjugate would provide an additional block to viral replication besides influencing Tat-mediated transactivation from the integrated HIV-1 LTR.

Since aliphatic diamines and polyamines have been shown to catalyze RNA hydrolysis,[37] we were interested to examine whether the neamine core of the conjugate

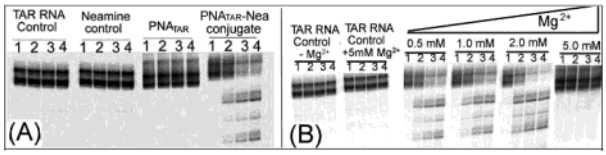


Figure 4. (A) Nuclease activity of conjugate **2.** The internally ^{32}P-labeled 96-mer HIV-1 TAR RNA (125 nM) was incubated with 4-fold excess of **2** or PNA **1** at pH 7.4. Lanes 1−4 in each set represent incubation at 25 °C for 0, 1, 3 and 5 h, respectively. Controls represent incubation of TAR RNA alone or with 4-fold excess of neamine. (B) Effect of Mg^{2+} on the nuclease activity of **2.** Experimental conditions were same except the addition of $MgCl_2$. Control represents incubation of TAR RNA alone in the absence or presence of 5 mM $MgCl_2$ under similar conditions.

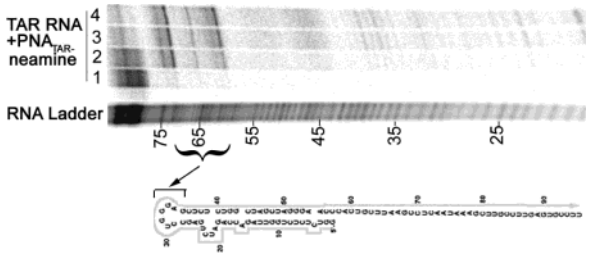


Figure 5. Conjugate **2** cleaves HIV-1 TAR RNA in the vicinity of its binding site. The internally ^{32}P-labeled 96-mer HIV-1 TAR RNA (125 nM) was incubated with 4-fold excess of **2** as described in Figure 4. Lanes 1−4 represent incubation for 0, 1, 3 and 5 h, respectively. The RNA ladders were generated from internally ^{32}P-labeled TAR under basic conditions. The arrow indicates the possible cleavage site on TAR RNA generating approximately 60−70 nucleotide long products.

carrying at pH 7 protonated and unprotonated amino groups[38,39] could also catalyze similar cleavage of the target RNA in the vicinity of PNA binding. Neomycin B has been shown to bind various RNAs[19] and accelerate the hydrolysis of the RNA dinucleotide ApA.[40] We incubated conjugate **2** with its target RNA at various time points. Results shown in Figure 4 strongly suggest that besides being sequence specific, the neamine moiety of the conjugate **2** can cleave the target RNA upon prolonged incubation at 25 °C. The cleavage activity was seen in the pH range of pH 7.0−8.0 with maximum activity at pH 7.4. (data not shown). No cleavage was seen when TAR RNA was incubated with either PNA or neamine alone under similar conditions. Significantly, this cleavage activity was unaffected at physiological concentrations (1−2 mM) of Mg^{2+} but strongly inhibited at higher concentrations of the divalent metal ion.

The cleavage site was mapped near the nucleotides at positions 30−39 in the stem-loop region of HIV-1 TAR in the proximity of the neamine core at the end of the PNA−RNA duplex, resulting a cleavage product of 60−70 mer (Figure 5). Although the mechanism remains to be elucidated, the cleavage could feasibly result from the presence of multiple amino groups on the neamine moiety with different pK_a values, ranging from 9.6 to 6.9[39,40] that cooperate for hydrolysis through acido-basic catalysis. Further investigation is required to understand the mechanistic details of this RNA cleavage reaction.

We examined the ability of conjugate **2** to block HIV-1 production when supplemented in the culture medium. For this purpose, we infected lymphocyte CEM cells with VSV-G pseudotyped HIV-1 virions carrying the firefly luciferase reporter gene. The infected cells were incubated with varying concentrations of **2** or naked PNA **1** during 48 h at 37 °C and lysed for measuring firefly luciferase activity. Results shown in Figure 6A indicate that inhibition of luciferase expression was observed at all concentrations of **2**. Approximately 50% inhibition was noted at 1 μM concentration of the conjugate. Supplementation of unconjugated PNA in the culture medium had no influence on luciferase gene expression. PNA **1** and neamine **3** did not inhibit HIV-1 replication even at concentrations greater than 5 μM in the culture medium.

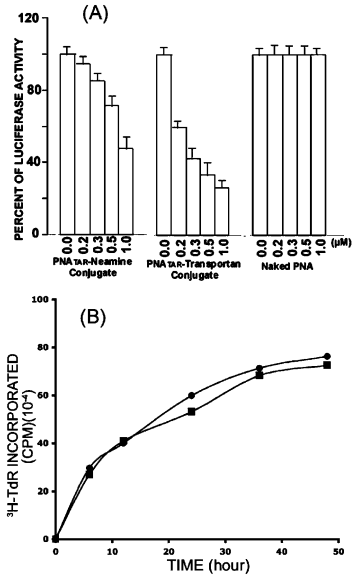


Figure 6. (A) Antiviral efficacy of conjugate **2**. CEM cells infected with pseudo-typed HIV-1 S1 strain in the presence of indicated concentrations of **2** or PNA **1**. Cells were harvested 48 h postinfection, lysed and assayed for luciferase activity. Anti-TAR PNA−transportan conjugate known to inhibit HIV-1 replication[14] was used as a positive control. The results are expressed as mean values along with standard deviation of three independent experiments. (B) Effect of **2** on cellular proliferation. [Methyl-^{3}H]thymidine (3H-TdR) incorporations in CEM cells were determined in the presence (■) and absence (●) of 5 mM conjugate. Results are represented as counts per min incorporated per mg of protein and are average value of two independent experiments.

To rule out the possibility that the observed dose-dependent inhibition of luciferase expression could be due to dose-dependent toxicity of the conjugate on HIV-1 infected cells, we carried out a [^{3}H]thymidine (TdR) incorporation study in the presence and absence of the conjugate. The HIV-1 infected cells were grown in the absence or presence of varying concentrations of the conjugate and supplemented with [^{3}H]thymidine. At indicated time points, aliquots were withdrawn and the total DNA was precipitated with TCA to determine the extent of thymidine incorporation. As shown in Figure 6B, the extent of [^{3}H]-TdR incorporation into cellular DNA in both the treated and untreated cells was similar at all time points, suggesting that cellular proliferation was not affected in the presence of the conjugate.

In conclusion, attachment of the neamine core to the terminal amino function of a PNA targeting the TAR region of the HIV-1 RNA genome allows cellular uptake of the PNA and results in potent inhibition of viral replication. The conjugate **2** also exhibits strong RNA cleavage activity. In regard to the recently reported PNA conjugate-based ribonuclease mimics,[9,10] the observed cleavage of TAR RNA in our system appears very efficient.

The observed antiviral activity of the conjugate may be mediated (i) through binding to the target RNA and perturbation of the transactivation process, (ii) by aborting reverse transcription in the TAR region or (iii) by inducing cleavage in the TAR region upon binding to the target RNA sequence. These results suggest that PNA−aminoglycoside conjugates could be exploited as potential antiviral agents. To study the mechanism of the antiviral effect and to further improve this effect, further modification of the conjugate and evaluation of cellular uptake in radiolabeled or fluorescently tagged forms is underway.

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Supporting Information Available: Experimental details for the synthesis of compounds **2**, **6** and **7** as well as biochemical and biological assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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