Prospects for antisense peptide nucleic acid (PNA) therapies for HIV

Virendra N. Pandey[1,*], **Alok Upadhyay**[1], and **Binay Chaubey**[2]

[1]Department of Biochemistry and Molecular Biology, UMDNJ-New Jersey Medical School, 185 South Orange Avenue, Newark, NJ 07103

[2]Centre for Advanced Studies Department of Botany, Calcutta University 35, Ballygunj Circular Road, Kolkata 700019

Abstract

Since the discovery and synthesis of a novel DNA mimic, peptide nucleic acid (PNA) in 1991, PNAs have attracted tremendous interest and have shown great promise as potential antisense drugs. **They have been used** extensively as tools for specific modulation of genes expression by targeting translation or transcription processes. This review discusses the present and future therapeutic potential of this class of compound as anti-HIV-1 drugs.

1. INTRODUCTION

In AIDS patients, genetic variation of the HIV-1 genome poses a major challenge to treatment regimens. The most commonly used anti-HIV-1 drugs target two key enzymes, protease and reverse transcriptase. The highly active anti-retroviral therapy (HAART), which also targets these key enzymes, has dramatically decreased AIDS-related mortality, lengthened the life expectancy, and improved the quality of life for infected individuals. Although these drugs have had great success in reducing the viral load in AIDS patients, the emergence of strains that are resistant to these drugs has made management of this disease particularly difficult.

Drug-resistant strains are usually recognized when the viral load does not fall even after the administration of combination therapies such as HAART. Furthermore, these therapies do not act on reservoirs of HIV in latently infected cells and therefore keep patients vulnerable to fresh eruptions of viral titer whenever the drug regimen is discontinued[1].

The major effort to establish a stable drug line to combat HIV-1 has focused on the accessory and regulatory proteins of HIV-1 as new drug targets. However, these targets have not yet been sufficiently explored; their functions in the cell and their modes of action in virus production and maturation are not well understood at the molecular level. Another major strategy, which has been worked on extensively, is aimed at inhibiting HIV-1 replication by targeting regulatory conserved and immutable sequences on the viral genome using antisense technology. These regulatory regions on the viral genome, which are essential for viral replication and gene expression, could be, individually or collectively, potential targets for the next generation of drug design and development. In this context, peptide nucleic acids (PNAs) have recently been explored as potential antisense agents for targeting these regulatory regions on the HIV-1 genome. PNAs are DNA mimics in which sugar phosphate backbone (Fig. 1) is replaced with N-(2- amino ethyl) glycine (aeg) units **[2].** This modification makes them highly

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*Corresponding Author Virendra N. Pandey Tel:973-972-0660 Fax:973-972-5594 pandey@umdnj.edu.

stable in the cellular compartment. In addition, PNAs show stronger binding affinity to their complementary RNA or DNA than do the respective RNA/DNA or DNA/DNA hybridizations [3].

2. The HIV-1 LTR and U5-PBS regions as attractive therapeutic targets of PNA

The first 1-333 nucleotides of HIV-1 genomic RNA comprise the nontranslated region, which contains several domains that are essential for viral replication (Fig. 2). The long terminal repeat (LTR) spans nucleotides 1-182 and consists of a unique 3′ region (U3), a repeat region (R), and a unique 5′ region (U5). This U3-R-U5 segment is located at both the ends of the viral genome. An 18-nucleotide-long domain that is required for the initiation of reverse transcription is located downstream of the U5 region of the 5′LTR; it spans nucleotides 183-201. This domain, the primer binding site (PBS), is complementary to the 3′ terminal 18 nucleotides of $tRNA_3^{Lys}$, which are involved in the initiation of reverse transcription [4,5]. Another important domain downstream of the PBS region is the dimerization initiation site, which spans nucleotides 235-281.This domain has been shown to have a dual role in genomic RNA packaging and in the second-strand transfer reaction during reverse transcription [6].

The 5′LTR of the proviral genome contains multiple cis-acting DNA target sequences for cellular transcription factors [7-9]. Also present in the LTR are various DNA elements, including the binding sites for nuclear factor NF-kB, NF-AT, and the stimulatory factor USF-1, which are required for stimulation of transcription in activated lymphocytes [10-12]. These cis-acting sequences can thus be successfully targeted to arrest viral multiplication. Other promising targets for drug intervention are the RNA binding regions for Rev and Tat regulatory proteins. The HIV-1 LTR contains a unique region, the TAR element, which spans nucleotides 1-60. The HIV protein Tat, which binds to the TAR region, is a potent transactivator of transcription that is required for virus infectivity [8,9,13-17]. The 3′ LTR, which functions as the transcriptional terminator, contains signals for polyadenylation of viral mRNA [18-20]. The DNA sequences, known as *att* sites, are important for the integration of proviral DNA into the host genome; they lie at the ends of the linear proviral DNA [21,22]. Mutation at the *att* site makes virus replication defective [23,24]. The *att* sites are located in the U3 and U5 region of LTR. Although the DNA sequences of the *att* site vary among different retroviruses, all of them have a CA dinucleotide located 2 bases from the 3′ end of both U3 and U5. Substitution of the conserved CA dinucleotide abolishes the integration reaction [25-27]. Another conserved sequence in the gag coding region at the site of initiation codon is an excellent target to block the translation of gag mRNA. This site has been effectively targeted by phosphorothioate oligonucleotides (GEM-91, GEM-92) complementary to this region [28-30]. These important sites on the viral genome are expected to be extremely stable since viruses with mutational changes in these regions are at risk of being eliminated from the viral population. PNAs targeting these sites on the viral genome could be excellent candidates for drug intervention.

3. PNA as a potential anti-HIV agent

The success of the anti-HIV activity of PNA depends on two major factors. The first factor is selection of a conserved region on the viral genome; the second is that the target region should be crucial to the HIV life cycle. Various studies have targeted the HIV-1 TAR region, a relatively conserved 59-mer (+1 to +59 nt) regulatory or enhancer sequence at the 5′ terminal of the HIV-1 genome. The viral protein Tat binds to this region and facilitates the recruitment of cellular factors cyclin T1 and CDK9, which phosphorylate the C-terminal domain of cellular RNA Pol II and facilitates its processivity - so that the complete viral genome is transcribed [31]. Inhibition of this transactivation process results in decreased viral production due to premature termination of transcription process. Therefore, HIV-1 TAR RNA is a potential

antisense drug target. The use of PNA against the TAR region has shown encouraging results [32-38].

Another relatively conserved and crucial region in the HIV genome is the U5 primer binding site (PBS), an 18-mer (+183 to +201 nt) sequence where the tRNA[lys] anneals and primes the synthesis of negative-strand strong-stop DNA. A 19-mer PNA targeted against the HIV-1 PBS blocked *in vitro* reverse transcription by binding to a preprimed RNA primer duplex [39]. An 18-mer PNA targeted to the PBS region blocked the initiation of reverse transcription by displacing the tRNA[Lys3] primer from the viral genome [40]. Interruption of this interaction blocks viral replication [41]. Similarly, PNA targeted against the polypurine tract (PPT) region of the viral genome could inhibit initiation of positive-strand strong-stop DNA synthesis [42]. Another conserved sequence near the 3′ end of the HIV-1 gag-pol transframe domain, when challenged by PNA, inhibited the production of virus up to 98.4% in chronically infected H-9 cells [43]. Another nonconserved region of the viral genome that has been targeted by PNA is the HIV-1 gag gene, which, upon binding to its target, completely blocked *in-vitro* transcription [44].

It is obvious from these studies that significant inhibition of viral replication was achieved by the antisense PNA targeted against different conserved regions of the viral genome, although none of them, individually, could inhibit 100% of viral replication. However, the PNA-based approach is still promising in that a cocktail of multiple PNAs can be used to target different conserved regions of the viral genome simultaneously, building up an additive antiviral effect. It is pertinent that PNA does not bind only to cognate mRNA, as do other antisense molecules or siRNAs, but can complex with the DNA duplex even in nondividing cells or the reservoir of cells with latent infection and exert an antigene effect [45]. Therefore, PNA can serve as an antisense as well as antigene agent against HIV-1.

4. Biodelivery of anti-HIV PNA

Since PNA/RNA hybrids are not substrates for RNase H [39,46], the antisense effect of PNA occurs mainly through blocking the function of its target by irreversible binding to the targeted sequences. Therefore, proficient biodelivery of PNA at a stoichiometric concentration to its target in the cell is of the utmost importance for its strong antisense effects. Different strategies for biodelivery have used naked or modified forms of PNA in cell culture systems or *in vivo* [47,48]. The major advance in biodelivery of PNA as therapeutic antisense was made by conjugating cell-transducing agents, including cell penetrating peptide (CPP). A number of reviews on CPP and their therapeutic application have recently been published [49-52]. A triphenyl-phosphonium-tagged PNA targeted to HIV-1 TAR was found to be efficiently taken up by cells and to block HIV-1 replication in cell culture [53]. In one unique study, PNA complementary to the HIV-1 TAR region was conjugated to neamine, a polycationic aminoglycoside. This conjugate was efficiently taken up by the cells and inhibited HIV-1 replication [36]. The PNA-Neamine conjugate targeted to HIV-1 TAR region displayed an endonuclease property that cleaved the targeted RNA sequence at the binding site [36,54].

The most efficient cellular uptake of anti-HIV-1 PNA was noted with the use of cell-penetrating peptides, including penetratin, transportan, and HIV-1 Tat-derived peptide as vehicle to deliver anti-HIV-1 PNA into cells. Uptake of CPP carrying bulky cargos has been shown via endosomal pathway specially with cationic CPP such as Tat and oligo-Arg [48,52,55], similar pathway has not been clearly established for less cationic CPP cargos such as PNA-penetratin conjugates. A number of devices for endosomal escape have been suggested including the use of lysosomotropic agents, membrane disruptive polymers and peptides [55]. When penetratin, a 16 residue peptide derived from the *Drosophila* homeodomain transcription factor antennapedia, is conjugated to PNA, its biological uptake is tremendously enhanced over that

of naked PNA [35,38,56]. Another CPPs, a chimeric transportan and 13-mer HIV-1 Tat peptide, has also been used successfully to improve the cellular uptake of anti-HIV-1 PNA [38,41,57, 58]. Transportan is a 27-residue chimeric cell-permeating peptide containing 13 amino acids from the amino terminus of the neuropeptide galanin and 14 amino acids from the amino terminus of the wasp venom toxin mastoparan [59]. When transportan was conjugated to a PNA analog targeted against the HIV-1 TAR region, the conjugate was rapidly internalized by the CEM cells, as well as chronically HIV-1 infected H-9 cells, and substantially inhibited HIV production [35,38,57]. This conjugate was distributed throughout the cell as observed by microscopy using a fluorescent TAMRA-tagged PNA [57]. This study was extended to evaluate the optimal length of the transportan residue essential for PNA uptake and its effective intracellular antisense activity. It was established that the full-length (27-mer) residue of transportan-PNA conjugate manifested maximum inhibition of viral replication in a cell culture system, although the uptake behavior of 27,22 and 21-mer Transportan-PNA conjugate was very close to that level [38]. The antiviral activity of anti-TAR PNA conjugated to different peptides had respective IC_{50} values of 400nM, 800nM, and 720nM for PNA transportan, PNA penetratin, and PNA tat in HIV-producing CEM cells. This clearly shows that PNA-Transportan conjugate had a better antiviral property in the cell culture system than did the other PNA conjugates. The exact reason for this difference in antiviral property of PNA conjugated to three different peptides is not well understood, but their uptake properties may be the major reason for this difference. Although the mechanisms of cellular uptake of PNA mediated through these peptide conjugates is not well explained, they are projected to adopt a cell-receptor-free mode of uptake [35,60,61].

4.1 Anti-HIV-1 PNAs conjugated to cell-penetrating peptides are potent antiviral and virucidal agents against HIV-1

An anti HIV-1 TAR PNA (PNA_{TAR}) conjugated with transportan peptide was shown to be rapidly taken up by cells, block Tat-mediated transactivation of HIV-1 transcription, and strongly inhibit HIV-1 production in chronically HIV-1-infected H9 cells [57]. It also has been found that the PNA_{TAR}-Transportan conjugate has potent virucidal activity. This conjugate rendered HIV-1 virions noninfectious on brief exposure, suggesting that it may have penetrated the host-derived viral membrane [35]. The anti-HIV-1 virucidal activity of this conjugate may be useful either in topical formulations to block HIV-1 infection or as a prophylactic agent for inactivation of HIV-1 in circulating plasma before its attachment and entry. Similar results were reported with anti-HIV-1 PNA conjugated with penetratin and tat-peptide [38].

The conjugates with penetratin, transportan and Tat peptides were shown to be the most effective as anti-HIV virucidal agents, with IC_{50} values in the range of 28 nM-37 nM [38]. The IC_{50} for inhibition of HIV-1 replication was lowest with PNA_{TAR}-Transportan-27 (0.4 μM), followed by PNA_{TAR}-Tat (0.72 μM) and PNA_{TAR}-Penetratin (0.8 μM). These results indicate that anti-HIV-1 PNAs conjugated with CPP not only inhibit HIV-1 replication *in vitro,* but also are potent virucidal agents.

4.2 Dual function of anti-TAR PNA-CPP conjugates

Besides blocking Tat-TAR interaction and down-regulation of HIV-1 gene expression [32, 34,57], anti-TAR PNA aborts the reverse transcription process catalyzed by viral reverse transcriptase [38]. HIV-1 RT is unable to displace the upstream PNA_{TAR} bound to the loop region of TAR RNA. Thus, the anti-TAR PNAs serve two purposes: preventing Tat- mediated transactivation of HIV-1 LTR, resulting in down-regulation of viral RNA production and gene expression, and interrupting reverse transcription by blocking the strand displacement reaction. Earlier, it was shown that the RNA strand in the PNA: RNA duplex is resistant to RNase H-mediated cleavage [39,46]. The loop region of TAR bound with PNA_{TAR} is also resistant to such cleavage. The inhibition of RNase-H by the anti-HIV-1 PNAs and the strand displacement

activities of HIV-1 RT should enhance the potential inhibitory effect of these molecules on RT and thereby halt viral replication. In fact, oligomeric PNAs targeted to multiple sites can be expected to have additive effects. In theory, this approach is an appealing way to adapt these molecules, not only as specific HIV inhibitors, but also as general anti-retroviral agents.

4.3 HIV-1 virions pretreated with PNA$_{TAR}$-CPP are blocked from carry out endogenous reverse transcription

It has been reported that PNA$_{TAR}$-CPP efficiently internalizes in the HIV-1 virion particle, binding to its target on the viral genome and blocking the synthesis of full-length (−) strand strong-stop DNA. When purified HIV-1 virions were incubated in the presence of ^{125}I-labeled conjugate and ultracentrifuged in discontinuous sucrose density gradient (10%-40%), a portion of the radioactivity sedimented at the bottom, corresponding to the sedimentation position of HIV-1 virions (Fig 3A;[35]. This indicates that the PNA-CPP conjugates are able to penetrate the membrane barrier of the virions. Moreover, the presence of 10% fetal calf serum in the incubation medium had no influence on internalization of the labeled conjugate in virions, indicating the absence of its nonspecific binding to serum proteins. When virions pretreated with PNA$_{TAR}$-CPP conjugate and separated from the conjugate on a 20% sucrose cushion were examined for endogenous reverse transcription activity, it was found that the majority of the RT products were blocked at the site of PNA$_{TAR}$-CPP binding in the TAR region (**Fig. 3B,** lanes 2 and 3). In controls without the conjugate or with scrambled PNA-Transportan conjugate, products larger than 400 bases were seen (lanes 1, 4, and 5). The predicted size of the tRNA$_3$[Lys] derived (−) ssDNA is 253 nucleotides. The two major bands migrating above the 400-nucleotide position represent products of strand transfer reaction.

4.4 PNA-CPP conjugates targeting the primer binding site (PBS) and upstream A-loop region inhibit initiation of endogenous reverse transcription

Among retroviruses, the synthesis of proviral DNA occurs by multi-step reverse transcription. HIV-1 exclusively uses tRNA$_3$[Lys] as the initiating primer; it anneals among the naturally occurring cellular tRNAs at the PBS and the upstream A-loop region in the 5′-UTR **[62].** Both PNA$_{PBS}$ and PNA$_{A-loop}$ efficiently displace the tRNA$_3$[Lys]-U5-PBS complex and inhibit the initiation of reverse transcription **[40,63].** Since HIV-1 virions pretreated with CPP conjugates of PNA$_{PBS}$ and PNA$_{A-loop}$ were rendered noninfectious, it was postulated that these conjugates may be readily internalized into the virion particles and bind to their target sequences on the viral genome. This hypothesis was tested by pretreating virion particles with varying concentrations of individual conjugates and then ultracentrifuging them through a 20% sucrose cushion.

When virion pellets were disrupted and assayed for endogenous reverse transcription activity, it was noted that initiation of reverse transcription was completely blocked in virion particles pretreated with both PNA$_{PBS}$-CPP and PNA$_{A-loop}$-CPP conjugates **[41].** The endogenous reverse transcription products from untreated virions or virions treated with scrambled PNA-CPP conjugate were larger than 490 nucleotides, indicating that strand transfer had taken place after synthesis of (−) strand strong-stop DNA. This suggested that the conjugates had permeated the virus envelope and bound to their cognate sequences on the viral RNA, thus preventing the initiation of reverse transcription. When HIV-1 virions were incubated with I^{125}-labeled PNA$_{TAR}$-CPP conjugate, the radioactive PNA-CPP conjugate co-sedimented with the virion particles **[35],** indicating that conjugates are internalized in the virion particles and render them noninfectious. As expected, both conjugates displayed potent virucidal activity against HIV-1 with IC$_{50}$ values of ~50 nM. The virucidal property of these conjugates suggests that a cocktail of anti-HIV-1 PNA-CPP conjugates targeting critical regions of the HIV-1 genome could serve as a prophylactic agent for inactivating HIV-1 virions after exposure to HIV-1 **[41].**

5. Neamine as an effective and stable vehicle to biodeliver Anti-HIV-1 PNAs

Neamine is a constituent of aminoglycoside antibiotics. These hydrophilic pseudo-oligosaccharides have several amino functions that are mostly protonated under physiological conditions. Neomycin B, an example of this class of antibiotics, contains a polycationic neamine moiety that not only binds the rev response element and TAR regions in HIV-1, but also blocks interaction of these regions with their respective transacting HIV-1 proteins, Rev and Tat [64,65];. The conjugation of anti-HIV-1 TAR PNA with neamine monomer at the 5-position of the neamine ring 1 enhanced its cellular uptake [36] (Fig. 4) and antiviral efficacy when supplemented in HIV-1 infected cell culture. It was also demonstrated that neamine-PNA conjugates are not toxic to cells and that the antiviral effect was due to inhibition of TAR-Tat interaction.

5.1 Anti-HIV-1 PNA-neamine conjugates display sequence-specific RNA cleavage activity

Since aliphatic diamines and polyamines catalyze RNA hydrolysis [66], we examined whether the neamine core of the conjugate carrying, at pH 7, protonated and unprotonated amino groups [67], could catalyze similar cleavage of target RNA in the vicinity of PNA_{TAR} binding. Neomycin B has been shown to bind various RNAs [68] and to accelerate the hydrolysis of an RNA dinucleoside phosphate, ApA [69]. When anti-TAR PNA-Neamine conjugate was incubated with its target RNA and DNA, the neamine moiety of the conjugate, besides being sequence-specific, specifically cleaved the target RNA but left corresponding DNA uncleaved **(Fig. 5A).** Optimum RNA cleavage occurred in the pH range of 7.0 to 7.4 [54]. No cleavage was seen with naked RNA_{TAR} or neamine alone under similar conditions. Significantly, this cleavage activity was unaffected at Mg^{2+} concentrations lower than 2 mM, but was strongly inhibited at higher concentrations of the divalent metal ion [36]. 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, finding a cleavage product of 60-70 mer (Fig. 5B).

A model of was proposed for the underlying mechanism of RNA cleavage involving amino groups in rings I and II of the neamine core (Fig. 6). Binding of the PNA moiety of the conjugate to the target RNA sequences brings the neamine moiety into close proximity to the phosphodiester backbone, so that the unprotonated amino group at the 3-position of ring I is within interacting distance with the 2 -OH of the ribose moiety. This interaction may increase the nucleophilic character of the vicinal 2 -oxygen atom through abstraction of a hydrogen atom by the amino group at the 3-position of ring I. The activated 2 -O may carry out a nucleophilic attack on the phosphorus atom of the phosphodiester group. If so, the protonated amino group at the 6-position in ring II may favor this attack by decreasing electron density in the phosphodiester group and donating its proton to the 5-oxygen atom of the following sugar, resulting in cleavage of the phosphodiester bond. This mechanism is supported by the finding that PNA attached to the 4-position in ring II was unable to cleave the target RNA [54]. This indicates that the position of conjugation of PNA with neamine is crucial for the observed ribonuclease activity.

6. Small lipophilic triphenylphosphonium (TPP) cation as an effective strategy for biodelivery of anti-HIV-1 PNA

In addition to using CPP and neamine as carriers, an easy-to-perform anti-HIV-1PNA biodelivery protocol was designed (Fig. 7A) based on attachment of PNA to the lipophilic triphenylphosphonium (TPP) cation through an intracellularly biodegradable carbamate linker containing a disulfide bridge [53]. The TPP moiety was selected for its ability to pass through a lipid bilayer, driven by membrane potential [70,71]. Once in the cytosol, the intracellular

glutathione pool rapidly reduces the disulfide bond, giving rise to the thiobutyltriphenyl cation (TBTP) and an unstable mercaptoethyloxycarbamate-PNA intermediate, leading to the release of free PNA (Fig. 7A).

This strategy has some similarities to that for promoting the intracellular release of anti-HIV active nucleoside monophosphates (Nu-MP) from phosphotriesters incorporating an enzymatically biodegradable protective agent **[72]**. In this new protocol, the releasable TPP carrier system and the N-extremity of PNA are linked via a carbamate group, which is stable in acidic or basic media and is relatively insensitive to enzymatic hydrolysis. It was found that the cellular uptake of fluo-PNA$_{TAR}$-TPP conjugate evaluated in CEM cells was 45%-50% within 6 h of incubation in RPMI medium containing 2% FCS (Fig. 7B). The IC$_{50}$ for antiviral activity of PNA$_{\textbf{TAR}}$-TPP conjugate was 1.0 □M.

7. Pharmacokinetic properties and toxicity of anti-HIV PNA-CPP conjugates

PNA$_{TAR}$-CPP conjugate in male Balb/C mice was pharmokinetically analyzed after administration through intraperitoneal or gavage routes **[73]**. The clearance profiles of the test compound from different organs and tissues as a function of time displayed a biphasic exponential pathway whereby part of the radioactivity cleared rapidly, while a significant portion of it was slowly released over a prolonged period.

The acute toxicity of this class of compounds was evaluated in mice by administering single IP doses of 100, 300, and 600 mg/kg of body weight. The 600 mg dose was lethal, killing all injected mice within 72 h. However, death did not occur after single doses of 100 and 300 mg/ kg, although mice experienced initial and transitory diarrhea, as well as loss of agility, which they quickly recovered. Thus, the LD$_{50}$ of the PNA$_{TAR}$-penetratin conjugate was determined to be ≤ 600-mg/kg body weight. Autopsies on dead mice revealed no gross findings that could be correlated with the lethal dose of the conjugate. It was presumed that the death of these mice might have been the result of diarrhea-induced dehydration caused by the high dose of the conjugate.

Repeat daily doses of 10 mg, 30 mg, and 100-mg/kg body weight were well tolerated by mice during 8 days of treatment. Daily doses of 100 mg/kg caused diarrhea during the first 4 days of treatment, after which mice showed distinct recovery from day 5 onward. During 8 weeks of follow-up, mice fully recuperated. Necropsies on mice given daily doses of 100 mg showed serenities in the spleen, liver, and kidney at the 9th day of treatment, but not after the follow-up period. Necropsies, clinical chemistry studies, and hematological parameters demonstrated normal function of the major organs and no irreversible damage to the mice. Therefore, the PNA-peptide conjugate should be completely nontoxic at therapeutic doses **[74]**.

Clinical blood chemistry analyses on the 9th and 60th days of the study showed normal levels of all values except those for ALT, which were marginally elevated (1.5-1.25-fold) in mice given a daily dose of 100 mg/kg body weight. Normal levels of alkaline phosphatase implied that there had been no significant damage to liver cells. Overall, these findings suggest the maintenance of normal cellular function at repeated high doses of the PNA$_{TAR}$-Penetratin conjugate. Most of the hematological parameters determined on the 9th and 60th days were within normal limits in all mice, suggesting that the anti-HIV-1 PNA-CPP conjugate had no deleterious effects on the health and functions of mouse organs. Similarly, the examination of bone-marrow aspirate revealed no abnormality in any mice. Differences in the ratio of myeloid to erythroblasts (M:E) were observed in all groups of mice only in 9th-day aspirate, suggesting that the PNA$_{TAR}$-Penetratin conjugate had no significant influence on the immune system **[74]**.

7.1 Naked PNA is immunologically inactive, while PNA-Penetratin conjugates are moderately immunogenic

The proliferation responses of splenocytes and lymph node cells from mice immunized with naked PNA$_{TAR}$. PNA$_{TAR}$-Penetratin conjugate or penetratin were determined by challenge with the same immunizing antigen. The responses obtained with naked PNA$_{TAR}$ as an immunizing and challenging antigen were negligible **[75].** Other studies have also suggested that, irrespective of the PNA sequences, naked PNAs are immunologically inert molecules **[68,69]**. However, the low proliferation responses noted with PNA$_{TAR}$-Penetratin conjugate could be due to the penetratin component of the conjugate. This premise was supported when lymph-node cells from mice immunized with penetratin or PNA$_{TAR}$-Penetratin conjugate were individually challenged with conjugate or its constituents, naked PNA$_{TAR}$ and penetratin (Fig. 8). The proliferation responses were significant when challenge was done with either penetratin or PNA-Penetratin conjugate, but not when it was done with naked PNA.

Analysis of the cytokine secretion profiles of lymph node cells from mice immunized with either PNA-Penetratin conjugate or penetratin and then challenged by the immunizing antigen yielded interesting results. The secretion of interleukin-2 (IL-2) by lymph node cells was the highest when they were challenged with either penetratin or PNA-Penetratin conjugate. Since the candidate compound, PNA$_{TAR}$-Penetratin conjugate, is a potent virucidal agent against HIV-1 and displays strong HIV-1 antiviral activity, the immunological response elicited by the conjugate with respect to the elevated production of IL-2 is expected to be beneficial to patients. Although IL-2 exerts its effects on many cell types, its most prominent effect is on the activation of T-lymphocytes **[76-79];**. IL-2 promotes proliferation of T lymphocytes and increases the count of mature T cells; it also slows the death of CD4+ T cells. HIV-1 infection not only decreases the CD4+ cell count and endogenous levels of IL-2, but also causes an increase in the dysfunction of existing CD4+ cells. These three deleterious effects of HIV-1 infection are potentially reversible with the administration of recombinant IL-2 **[80]**. Clinical trials have shown that HIV-1 patients undergoing antiretroviral therapy with IL-2 supplement show both significant increases in the number of their CD4+ cells and significant decreases in HIV/AIDS-related morbidity as compared with patients receiving the same therapy without IL-2 supplement **[81-88].**

In addition to IL-2, interleukin-12 (IL-12), interferon-gamma (IFN-γ;), and tumor necrosis factor-alpha (TNF-α) were produced by lymph node cells from immunized mice. IL-12 stimulates the growth and function of T cells and the production of IFN-γ and TNF-α. Both IL-12 and IL-2 are important cytokines in the regulation of a cell-mediated immune response, with IL-2 being responsible for stimulating the proliferation of T cells while IL-12 promotes differentiation of the antigen-activated CD4+ T cells into the TH1 cell type **[89]**. The production of pro-inflammatory cytokines (IL-2, IFN-γ, TNF-α), but not anti-inflammatory cytokines (IL-4, IL-10) from lymph node cells stimulated with penetratin and PNA$_{TAR}$-Penetratin conjugate is consistent with TH1 cell differentiation, which supports cell-mediated immune responses (e.g., cytotoxic T lymphocytes, natural killer cells, activated macrophages) that would benefit patients with HIV-1 infection.

8. Current approach to developing virucidal vaginal gel formulations of anti-HIV-1 PNA-CPP conjugates

The majority of HIV-1 infections are due to heterosexual contact **[90]**, although the highest risk for HIV-1 transmission occurs with receptive anal intercourse **[91]**. The semen from HIV-1 infected men **[92,93]** and cervical mucus from HIV-1 infected women contain both cell-free virus and HIV-1 infected cells **[94]**. Therefore, sexual transmission of HIV-1 can occur by both cell-free HIV-1 virions and HIV-1 infected cells. However, infection can be prevented by using

either mechanical barriers such as condoms or an effective virucidal vaginal gel formulation. As of July 2008, six anti-HIV-1 vaginal gel formulations were undergoing clinical trials in different countries: PRO 2000/5, Tenofovir, SPL7013 (VivaGel), Dapivirine (TMC120), HEC/ CS/N-9, Emollient, and UC-781. Of these, four are undergoing phase 1 clinical trials; two (PRO2000/5 and Tenofovir) are in phase 2/3 clinical trials.

Efforts are under way to develop a potential vaginal gel formulation using a cocktail of nontoxic anti-HIV-1 PNA-CPP conjugates, which have been shown to be potently virucidal against HIV-1. The most important pharmacological properties of anti-HIV-1 PNA-CPP conjugates are their long biological half-life and nontoxic characteristics, even at high unphysiological doses of 100-300 mg/kg body weight **[74]**. Moreover, these conjugates are potently virucidal even in subnanomolar concentrations. Because these conjugates are capable of efficient internalization in virion particles and in uninfected or infected cells, they are expected to block sexual transmission of HIV-1 by inactivating virion particles in semen and by interfering with virus replication or production in HIV-1-infected cells in both semen and cervical mucus from HIV-1-infected individuals. A virucidal gel can be formulated with a cocktail of anti-HIV-1 PNA-CPP conjugates targeting HIV-1 TAR, the primer-binding site, and its upstream A-loop region, the dimerization site of HIV-1 RNA. It therefore should offer a multipronged effect, blocking every phase of the HIV-1 life cycle.

9. Future strategies for enhancing the selective biodelivery of ani-HIV1 PNA to CD4+ T lymphocytes and macrophages

The clinical potential of current anti-HIV-1 drugs has been limited not only by their toxicity and the development of drug-resistant strains of the virus, but also by nonspecific systemic delivery of the drugs. The therapeutic potential of these drugs could be greatly increased if they could be selectively delivered into T-lymphocytes and macrophages, which are, respectively, the targets and reservoirs for HIV-1 infection and replication. Although anti-HIV-1 PNA-CPP conjugates display potent antiviral and virucidal activities, they penetrate all organs and cell types and distribute within them with equal efficiency. Effective strategies for biodelivery of anti-HIV-1 PNAs specifically into T-lymphocytes and macrophages would be an ideal approach to block HIV-1 infection and replication.

9.1 The use of glycoconjugates containing mannose clusters as potent vehicles to deliver anti-HIV-1 PNA to macrophages

The mannose receptor (MR) is an endocytic receptor of macrophages and endothelial cell subsets that selectively bind and internalize both endogenous and foreign glycoconjugates **[95,96]**. These carbohydrates, which are present on the surface of many microorganisms, enable the MR to bind and internalize them during phagocytosis. MRs, which are expressed on human macrophages and cultured dendritic cells, are attractive points for specific drug delivery to these cells. Therefore, designing cell-recognizable glycoconjugates of anti-HIV-1 PNA having a cluster of mannose will be highly desirable for their specific delivery to macrophages.

9.2 Formylated oligopeptide as a potent chemoattractant for selective delivery of anti-HIV-1 PNA to macrophages

The movement of monocytes or macrophages to various tissue sites is greatly influenced by various chemotactic substances, including lymphokines and bacterially derived factors, which are responsible for macrophage migration toward a site of bacterial invasion **[97]**. It has long been known that small synthetic N-formylated oligopeptides, which are structurally related to bacterially derived products, are potent chemoattractants for macrophages **[97]**. A small formylated peptide, N-formyl-Nle-Leu-Phe-Nle-Tyr-Lys, is a potent chemoattractant with

high receptor-binding affinity. The number of receptors on each macrophage was determined to be 120,000, with a dissociation constant of 1.3 nM **[98,99]**. Binding of chemoattractant peptides to macrophages at physiological temperature is highly specific, rapid, and saturable. After time- and temperature-dependent binding, the receptor ligand complex is altered and is no longer reversible since the ligand is rapidly internalized into the cells **[98,99]**. Use of this unique and specific interaction between chemoattractant formylated peptides and macrophages can be exploited to deliver anti-HIV-1 PNA selectively into macrophages.

9.3 Use of the V3-loop peptide of gp120 as the navigator for anti-HIV-1 PNA to ferret out lymphocytes and macrophages

The V3-loop region of HIV-1 gp120 is critical for HIV-1 infection. In addition to interacting with the CD4 receptor and coreceptor, gp120 interacts specifically with certain surface glycosphingolipids (GSL) such as the globotriaosylceramide (Gb3) and monosialoganglioside (GM3) of lymphocytes and macrophages. These interactions are crucial for viral entry and fusion **[100,101]**. CD4-independent HIV-1 infection of human colonic epithelial cells (HT-29) involves the use of galactosylceramide (GalCer) as an alternate receptor for gp120 **[102]**. A small peptide corresponding to the central 15-amino-acid region of the V3-loop (RIQRGPGRAFVTIGK) of HIV-1 gp120 exhibits specific binding to host cell membrane GSL-like GalCer, GM3, and Gb3, and effectively competes with gp120 from various HIV-1 strains **[103]**. This binding specificity of the V-3 loop peptide can be an attractive strategy to navigate anti-HIV-1 PNA specifically to T-lymphocytes, macrophages, and colonic epithelial cells.

9.5 A synthetic peptide corresponding to variable regions of an anti-CD4 monoclonal antibody specifically binds to extracellular domain D1 of CD4

The CD4 receptor is a transmembrane glycoprotein on thymocytes, T-cells, and macrophages. CD4, a member of the immunoglobulin gene superfamily, consists of four extracellular domains (D1-D4) **[104]**. The loop region of the D1 domain of CD4 has been identified as the primary binding site for HIV-1 gp120, which, in association with chemokine receptors, facilitates HIV-1 entry into cells **[105-107]**. A peptide derived from the variable region of anti-CD4 monoclonal antibody (GGVLWSRRGDFD) specifically binds to the D1 loop of CD4 with an affinity of 1.6 nM Kd **[108]**. Attaching this peptide to anti-HIV-1 PNAs should greatly enhance their specificity to targeted cells.

10. Conclusion

The sequestering of critical regulatory sequences in the viral genome by sequence-specific PNA conjugated with specially designed peptide and mannose clusters that specifically target T-lymphocytes, macrophages, and rectal and vaginal epithelial cells has great potential to block HIV infection and replication. This is an important new strategy for therapeutic intervention. The armamentarium of antiretroviral agents targeting one or more steps in the replicative cycle of HIV is likely to grow in the near future. It is possible that multiple steps in the viral life cycle can be targeted for new antiretroviral therapy. In this context, the virucidal and antiviral activities of PNA-CPP conjugates targeting critical regions of the HIV-1 genome hold enormous promise with regard to the development of effective virucidal vaginal gel formulations to stop HIV-1 transmission at the earliest stages of infection. The most compelling aspect of this approach is that, upon exposure to PNA-CPP conjugates targeted against critical regions of the viral genome, HIV-1 in the plasma can be inactivated before its entry into cells. Penetrating the virion particles, the PNA-CPP conjugates render them replication-incompetent. These conjugates have great potential as prophylactic agents to block HIV-1 infection. Studies on the pharmacokinetics and toxicity of these compounds have clearly demonstrated that they

are nontoxic and have prolonged clearance profiles. These attributes should form the basis for determining their appropriate doses for preclinical evaluation.

11. Expert Opinion

PNAs have been in the news since their discovery in 1991. However, the initial excitement about the potential of this new class of compounds as potential antisense molecules has been dampened by their poor cellular uptake. Many strategies have now been developed to improve their cellular uptake, with cell-penetrating peptides proving to be the most effective biodelivery vehicle. Although efficient cellular uptake occurs with anti-HIV PNA-CPP in cell culture systems, a detailed study of their uptake efficacy in primary cells and in-vivo systems remains to be determined. The CPP conjugated with PNA via an amide bond is most stable in cells and does not interfere with target specificity. One drawback of this approach is expected to be the route of administration. PNA-CPP conjugates must be administered either intraperitoneally or intravenously since gavage of PNA-CPP conjugate is not efficient due to cleavage of peptide in the gut. The D form of CPP peptide should enhance the stability of anti-HIV-1 PNA-CPP conjugates in the gut. Gavage of naked PNA is not encouraging because the PNA remains in the stomach for a long time. A strategy to enhance oral biodelivery of PNAs will certainly improve their therapeutic potential. In this context, anti-HIV PNA-Neamine conjugates are promising, being stable in the gut environment and relatively effectively taken up when administered orally (unpublished results).

One detailed toxicity study of an anti-HIV-1 PNA CPP conjugate is encouraging because it showed that this conjugate is almost nontoxic to mice at multiple doses of 100 mg/kg of body weight, with an LD_{50} between 300-600 mg/kg body weight [74]. Because the body-to-surface area of a mouse ($0.0066m^2$:0.02kg) is 12-fold higher than that of humans ($1.6 m^2$/60kg) [109], the effective human dosing concentrations of PNA-CPP conjugates are expected to be 10-12-fold lower than those for mice. These small mammals also eliminate drugs faster than large mammals do [110]. One point of concern is that cellular uptake of PNA-CPP conjugates or PNA-Neamine conjugates is not cell-specific. The clinical potential of anti-HIV-1 PNA could be enhanced several-fold if it were selectively delivered into CD4-positive T cells and macrophages, which are, respectively, the targets and reservoirs for HIV-1 infection and replication. Therefore, alternate strategies are needed for their specific delivery into T-lymphocytes and macrophages, which support CD4-dependent HIV infection, and into human colonic epithelial cells, which use GalCer, a major glycosphingolipid expressed by these cells, for HIV-1 infection. Optimization of their biodelivery by conjugating these anti-HIV-1 PNAs to specially designed peptide and mannose clusters aimed specifically at T-lymphocytes, macrophages, and human colonic epithelial cells would offer a logical and promising means of blocking HIV replication in its sanctuaries.

Women, who now account for nearly 60% of newly HIV-infected adults worldwide, most often acquire HIV through heterosexual exposure [111]. The semen from HIV-1 infected men [92, 93,112] and cervical mucus from HIV-1 infected women contain both cell-free virus and HIV-1 infected cells [94]. Therefore, sexual transmission of HIV-1 can occur by both cell-free HIV-1 virions and HIV-1 infected cells. Targeting the earliest stages of infection offers the best prospect for preventing or containing infection. The virucidal and antiviral properties of anti-HIV-1 PNA-CPP conjugates could be exploited in the development of a potent virucidal vaginal gel formulation with components known to have excellent rectal and vaginal tolerance and strong bioadhesive properties.

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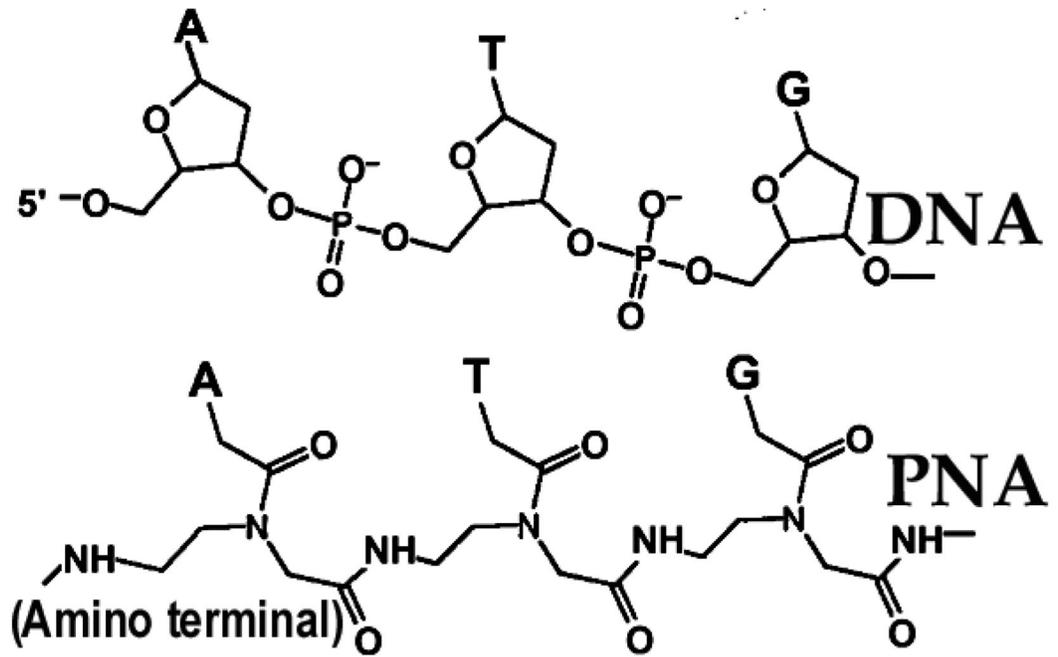
Fig.1.
Comparison of Chemical Structure for PNA and DNA: In the structure of PNA, bases (A, T, G, C) are linked with polyamide backbone as against sugar phosphate backbone in DNA.
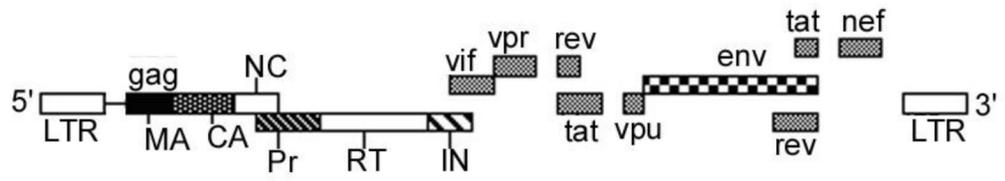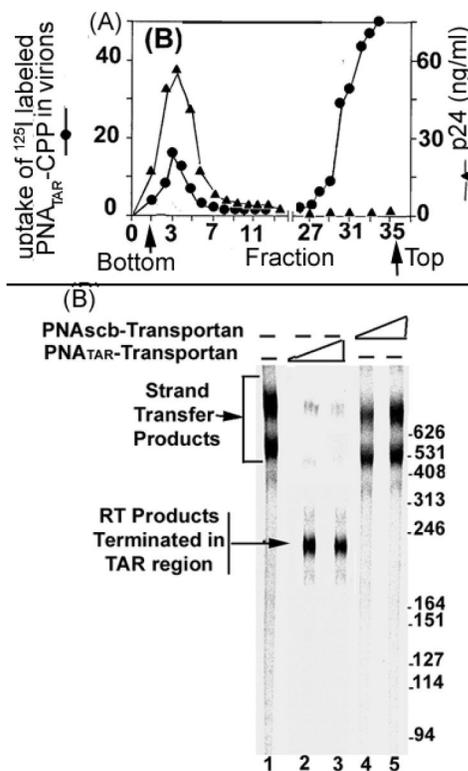
Fig.2.
Organization of HIV-1 genome

Fig 3.
(A) Internalization of PNA_{TAR}-CPP in HIV-1 virions. The HIV-1 virions incubated with I^{125}-labeled PNA_{TAR}-CPP conjugate was subjected to discontinuous sucrose density gradient ultracentrifugation. Fractions collected from the bottom and analyzed for p24 antigen and for radioactivity **[35]**.
(B) Abortive endogenous reverse transcription in HIV-1 virions pretreated with PNA_{TAR}-CPP conjugate [35]. Lane1, untreated HIV-1 virions; lanes 2 and 3, pre-treated with 1 μM and 2.5 μM of PNA_{TAR} Transportan conjugate, respectively; lanes 4 and 5, pre-treated with scrambled PNA-Transportan conjugate at 2 μM and 5 μM concentrations, respectively.
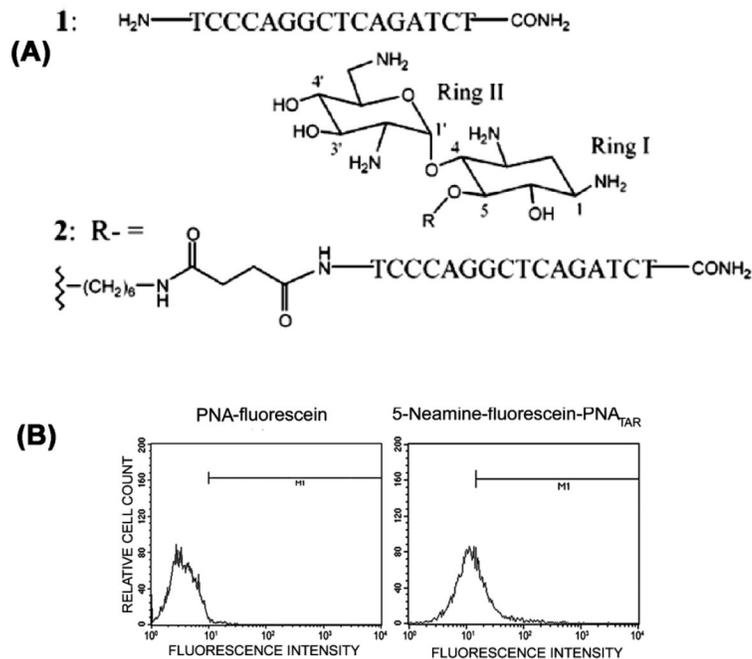
Fig. 4.
(A). Structure of the anti-TAR PNA- neamine conjugate and the aminoglycosides neamine.
(B) Cellular uptake of PNA-Neamine conjugate. Lymphocytes incubated with 2 μM conjugate at 37°C in complete RPMI medium and uptake of the conjugate in the cells was examined by FACscan **[33,47].**
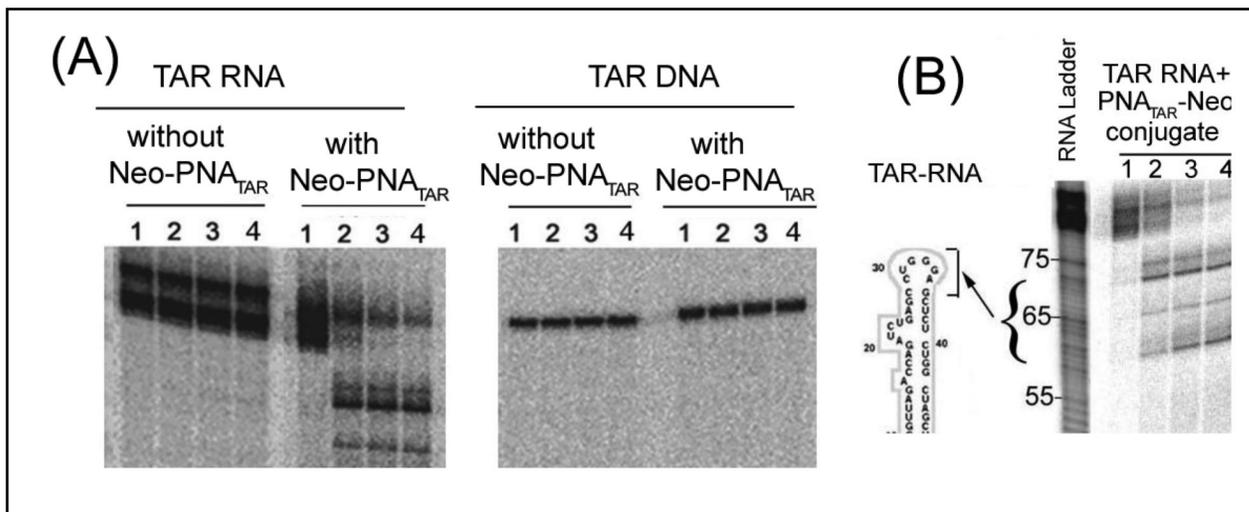
Figure 5.
(A) PNA$_{TAR}$.Neamine specifically cleaves the target RNA but not the corresponding DNA. Internally ^{32}P-labeled TAR RNA or 5′ ^{32}P-labeled DNA incubated in the presence or absence of 400 nM 5-neamine-PNA$_{TAR}$ conjugate at 25°C. Lanes 1 through 4 in each panel show the results after incubation periods of 0, 1, 3, and 5 h, respectively **[54]**.
(B). PNA$_{TAR}$ Neamine Conjugate cleaves HIV-1 TAR RNA in the vicinity of its binding site. The internally ^{32}P-labeled HIV-1 TAR RNA incubated without or with 400 nM of the conjugate at room temperature. Lanes 1-4 represent incubation for 0, 1, 3 and 5 h, respectively. The arrow indicates the possible cleavage site on TAR RNA generating approximately 60-70 nucleotide long products **[33]**.
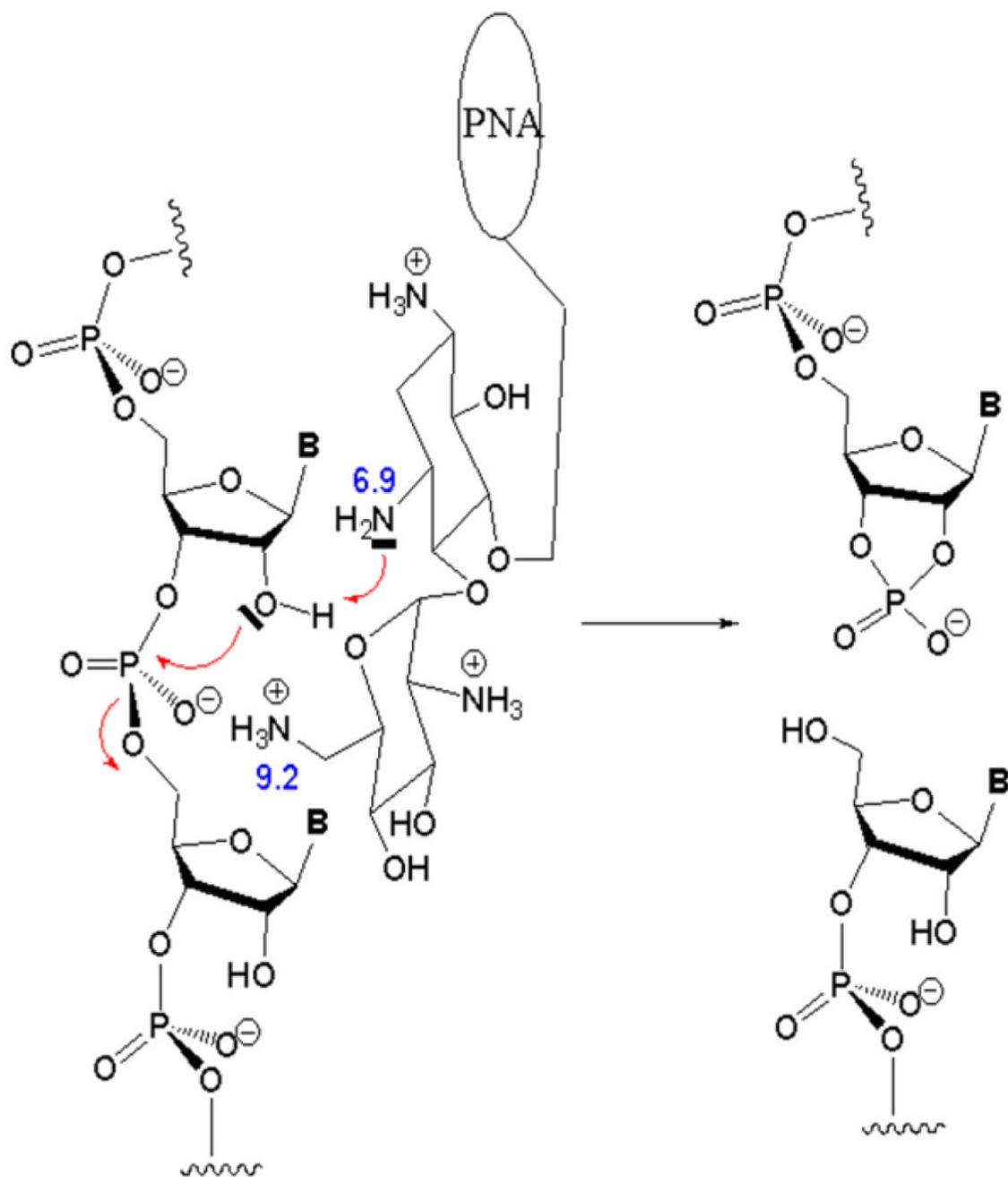
FIG. 6.
Proposed mechanism of PNA-Neamine conjugate-mediated cleavage of transactivation response element (TAR) RNA. The PNA attached to the 5-position of ring I of the neamine moiety through an 11-atom linker is shown as a circle. The numbers shown are the pKa value of amino groups at positions 3 and 6 of ring 1 and ring 6 of the neamine moiety. The deprotonated amino group at position 3 of ring I is shown extracting a proton from the 2 OH group of the ribose moiety of the RNA backbone. The activated 2 O is shown interacting intra-molecularly with a phosphorus atom, while the protonated amino group at the 6 -position in ring II is shown interacting with the negatively charged oxygen atom of the phosphate group.

The arrows indicate the direction of interaction leading to final cleavage of the phosphodiester bond **[54]**.
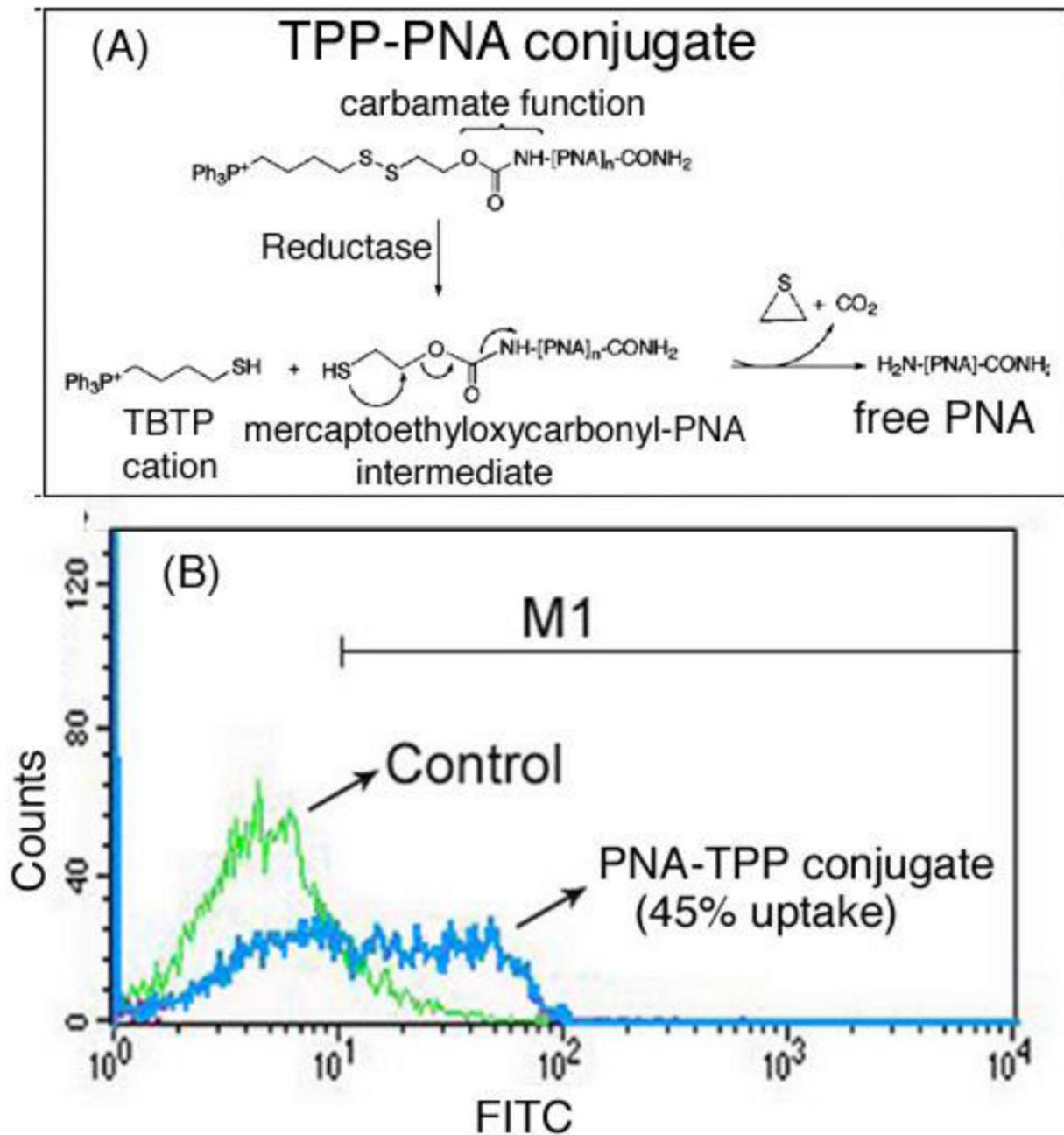
Figure 7.
(A) Synthesis of PNA-TPP conjugate and mechanism of intracellular release of free PNA following uptake. **(B)** Flow-cytometry of uptake of TPP-PNA conjugate **[53]**.
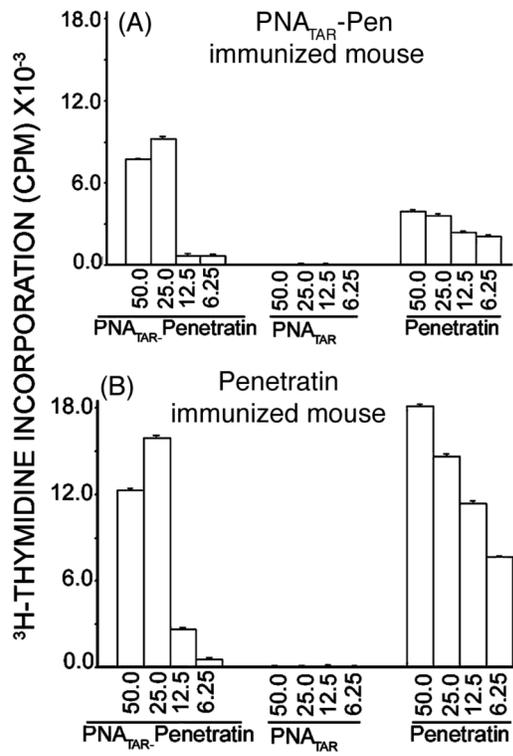
Figure 8.
Proliferation responses of lymph node cells from the conjugate-immunized **(A)** or penetratin immunized **(B)** mouse challenged with naked PNA, penetratin and the conjugate **[67]**.