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Therapeutic Potential of Aptamer-siRNA Conjugates for Treatment of HIV-1

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Abstract

Therapeutic strategies designed to treat HIV infection with combinations of antiviral drugs have proven to be the best approach for slowing the progression to AIDS. Despite the great success of highly active antiretroviral therapy (HAART), drug resistance and toxicity issues still remain a concern for some individuals. Therefore, alternative therapeutic strategies need to be developed to overcome these limitations. Nucleic acid-based therapeutics have been considered as an alternative to the currently used antivirals. In this regard, RNA interference (RNAi) can function as a gene-specific therapeutic option for controlling HIV-1 replication. Another type of therapeutic nucleic acid – aptamers – shows promise as a new and potent class of anti-HIV agent and can additionally function as a cell-type-specific delivery vehicle for targeted RNAi. The combined use of small interfering RNA (siRNAs) and aptamers could effectively block viral replication and prevent the emergence of resistant variants. In this review, we recapitulate recent progress and the therapeutic potential of aptamer-siRNA conjugates in the treatment of HIV infection.

Keywords

Drug-targeting delivery-systems; HIV-1-infections; Nucleotide-aptamers; RNA-interference; Small-interfering-RNA

1. Introduction

Currently, there is no cure for HIV/AIDS, but a variety of antiretroviral drugs that act on different stages of the HIV life-cycle can be used in combination to control the virus. In typical highly active antiretroviral therapy (HAART) treatment, three different drugs are taken in combination to avoid creating strains of HIV that are resistant to a single drug. Some fixed-dose combinations and preferred regimens are introduced in clinical treatment. Although HAART treatment for HIV/AIDS has been therapeutically effective in the majority of patients, severe side effects, toxicities, compliance problems, and the emergence of drug-resistant mutants complicate the use of this therapy.^[1] Currently, the global

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epidemic of infection by HIV-1 has created an urgent need for innovative approaches for treatment of HIV/AIDS. In this regard, nucleic acid-based therapeutics have been considered as an alternative to the chemical antivirals to overcome these limitations.^[2,3]

The concept of nucleic acid-based therapeutics is not a new one and has captured the attention of scientists for a long time. In the early attempts to develop nucleic acid therapeutics for HIV-1, the researchers mainly focused on ribozymes and RNA decoys or mimetics/aptamers.^[4–6] For example, RNA decoys against TAR^[7,8] (trans-activating response region), RBE (rev-binding element) and RRE (rev responsive element); ribozymes targeting Gag (group-specific antigen), Env (envelope) and CCR5 (C-C chemokine receptor type 5); and aptamers specific for HIV-1 transactivators (Rev, Tat), RT (reverse transcriptase),^[9,10] INT (integrase), RNaseH, and HIV-1 envelop gp120,^[11–15] etc. were investigated. Most recently, with the advent of RNA interference (RNAi) technology for specifically silencing target genes,^[16,17] RNAi-based therapeutics such as small interfering RNAs (siRNAs) have unique therapeutic potential for the treatment of HIV-1 infection since the entire genome of the virus is a target.^[18,19] To date, numerous siRNAs against a number of HIV-1 genes and cellular receptors have demonstrated efficient inhibition of viral replication both *in vitro* and *in vivo*.^[20,21] For example, siRNAs specific to HIV-1 encoded genes (*tat*, *rev*) were highly effective *in vitro*.^[22] Several well-known host factors that are essential for viral entry, including the CD4 receptor and co-receptor CCR5, represent attractive cellular targets for siRNA-mediated inhibition of viral replication in cell culture and in humanized animal models.^[19]

In addition to their possible utility as stand-alone inhibitors, the combinatorial use of various antiviral nucleic acids could be more efficacious in blocking viral replication and preventing the emergence of resistant variants.^[23–27] For example, a single lentiviral vector containing a triple combination of an shRNA targeting HIV-1 *tat/rev* mRNAs with an anti-CCR5 hammerhead ribozyme and a nucleolar-localizing TAR RNA decoy has been developed to efficiently suppress HIV-1 replication over 42 days, and the triple combination was more effective than a single anti-*tat/rev* shRNA or double combinations of the shRNA/ribozyme or decoy.^[22] Another class of therapeutic nucleic acids – aptamers – shows promise as a potential candidate for targeted siRNA delivery. By functionalizing the cell-specific aptamers with therapeutic agents, the cellular uptake is enhanced, thereby improving the therapeutic efficacy. Most recently, two aptamers raised against CD4 (cluster of differentiation 4) and HIV-1 glycoprotein gp120 have been harnessed for targeting delivery of anti-HIV siRNAs *in vitro* as well as in humanized mice. For instance, anti-HIV envelope-specific aptamers have been successfully combined with anti-HIV siRNAs to achieve a dual-inhibitory drug that is able to deliver siRNAs selectively to HIV-infected cells for target mRNA knockdown as well as inhibiting viral entry via blocking of the envelope interaction with the CD4 receptor.^[28–30] These recent efforts demonstrate that nucleic acid drugs can be successfully used in combinations resulting in efficacious treatments for HIV infection. Therefore, the objective of this narrative is to provide a brief overview of the development of RNAi therapeutics and of nucleic acid aptamers, placing particular emphasis on the development of aptamer-mediated siRNA delivery for targeted HIV therapy.

2. Progress and Challenges of RNA Interference (RNAi)-Based Antiviral Therapeutics

RNAi is an evolutionarily conserved process found in organisms ranging from fission yeast to man.^[16] By taking advantage of the cell's endogenous RNAi machinery, siRNA duplexes 21–27 nt in length can guide the sequence-specific degradation of a targeted cellular mRNA.^[17,31] With the exquisite specificity and high efficacy of RNAi, the prospect of using siRNAs for treating human viral infections and other diseases remains attractive.^[32,33]

HIV-1 became one of the first infectious agents targeted by RNAi due to its well understood life-cycle and pattern of gene expression. However, several major obstacles for the long-term use of RNAi against chronic HIV-1 infection are (i) viral escape from the RNAi mechanism;^[23,26] (ii) inefficient delivery/poor cellular uptake in the target cells or tissue; and (iii) suboptimal gene silencing activity owing to instability and poor bioavailability of siRNA agents.^[34]

Development of viral resistance to drugs, a result of the generation of viral escape mutants, is a common setback with HIV-1 therapies. It has been demonstrated that a single nucleotide mutation in a critical position within the target sequence relative to the siRNA site of interaction can diminish and even eliminate RNAi inhibition. Therefore, one of the crucial concerns in achieving RNAi efficacy is mitigating viral escape from RNAi. Extensive efforts have been undertaken to identify cellular proteins required for HIV-1 infection and replication^[35] and to develop combinatorial therapies which include host dependency factors along with the virus itself.^[36,37] In similarity to the conventional HAART approach, the combinatorial use of multiple RNAi effectors targeting separate multiple viral targets or HIV host dependency factors has been shown to mitigate and even prevent viral escape, thereby achieving prolonged viral suppression. Three independent siRNA-screening studies have identified hundreds of host factors that are critical for HIV-1 replication, which were not previously known to be required by the virus.^[38–41]

In addition to identification of new anti-HIV targets and combinations of multiple anti-HIV siRNAs, there have also been concerted efforts to develop safe and effective siRNA delivery systems, which are key for the clinical translation of RNAi.^[33–34] The average size of a single siRNA molecule is below 10 nm and non-formulated siRNAs administered directly into the blood are rapidly excreted by renal filtration. SiRNAs that lack some carriers do not pass through the blood vessel endothelial wall and the multiple tissue barriers.^[42,43] Furthermore, the negatively charged siRNAs do not readily traverse cellular membranes and enter into cytoplasm where the siRNAs can be incorporated into the RNA-induced silencing complex (RISC) for RNAi.^[44] Therefore, a proper delivery formulation is necessary to enhance siRNA stability and achieve cellular uptake and consequent therapeutic efficacy. To this point, a targeted delivery system is highly desired. For example, by functionalizing the cell-specific nucleic acid aptamers with siRNAs, the cellular uptake is enhanced and the local concentration of the siRNA drugs in the targeted cells or tissues is increased, thereby improving the therapeutic potency.

3. Development and Application of Cell-Specific Aptamers

Since the first reports of aptamers in 1990, an increasing number of aptamers have been screened and tested as therapeutic agents. As nucleic acids that assume specific and stable three-dimensional shapes, aptamers can be evolved *in vitro* under conditions which provide highly specific, tight binding to targeted molecules.^[45–47] The low nanomolar dissociation constants and specificity of aptamers to their targets make them attractive for the treatment of HIV.^[4–5] More than ten different aptamers have been investigated for use against HIV and some of them have been evaluated in animal models. For example, anti-RT (reverse transcriptase) aptamers that interact tightly with the polymerase active site have shown inhibition of viral replication in cell culture.^[9,48] Moreover, RNA aptamers that block the interaction of HIV-1 gp120 envelope with the CD4 receptor as well as aptamers targeting CD4 itself have been isolated *in vitro* and effectively neutralized R4 and R5 strains of HIV-1 in cell culture and in humanized mouse models.^[12,14,29,49] In the following section, we will focus on these two specific aptamers that have proven to inhibit viral infection and replication *in vitro* and *in vivo*.

Apart from therapeutic applications, aptamers raised against cell membrane receptors have emerged as promising delivery agents to target a distinct disease or tissue in a cell-type-specific manner.^[50,51] The aptamer can recognize a cell-surface receptor that undergoes either constitutive or ligand-mediated internalization, such as via a clathrin-mediated endocytic pathway.^[42,43] Currently, through either traditional purified membrane protein-based SELEX (systematic evolution of ligands by exponential enrichment)^[52–55] or the more recently emerging whole-cell-based SELEX procedure,^[56–58] an increasing number of cell-specific DNA or RNA aptamers have been isolated against cell surface biomarkers or receptors for specific cell type recognition. Several aptamers have been successfully harnessed for targeted delivery of siRNA molecules *in vitro* as well as *in vivo*.^[59] The first example of cell-specific aptamers used for targeted siRNA delivery are RNA aptamers that bind the prostate-specific membrane antigen (PSMA).^[60–63] PSMA is a trans-membrane protein that is highly expressed in human prostate cancer and vascular endothelium that provides a paradigm for aptamer-mediated siRNA delivery.^[64]

It is well-known that HIV-1 infection is initiated by the interactions between the human cell surface receptor CD4 and the envelope glycoprotein gp120 of HIV, subsequently leading to fusion of the virus with the target cell membrane.^[65–69] Thus, HIV-1 entry into its target cells represents an attractive target for new anti-HIV-1 drug development. Aptamers specific to either HIV-1 gp120 or the CD4 receptor have been generated with the use of a BIAcore biosensor system,^[11,12] conventional nitrocellulose filter binding,^[29] or Sepharose bead capture.^[49] The selected aptamers can specifically bind to and be rapidly internalized into CD4⁺ T-cells or HIV-1 infected cells. They have been conjugated with siRNAs targeting either cellular components of the virus itself for specific siRNA delivery and viral suppression.

4. Cell-Specific Aptamer-Functionalized RNAi in HIV Therapy

Through either covalent conjugation or physical assembly, different siRNA molecules have been successfully functionalized with these cell-specific aptamers against HIV-1 gp120 and the CD4 receptor to achieve targeted RNAi efficacy (figure 1a and 1b), which relies on specific interactions between the aptamer and its receptor expressed on the targeted cells or tissue. In order to achieve efficient RNAi activity, aptamer-siRNA conjugates must be successfully internalized and released into the cytoplasm where they can encounter the RNAi machinery. As shown in figure 2, cell-specific aptamer-directed recognition events consequently increase the local concentration of the drug in the targeted cells or tissue. After the aptamer-receptor binding, the cellular receptors are readily internalized and rapidly re-expressed on the cell surface to allow repeated targeting and internalization. Following cellular uptake, the endocytic vesicles formed by the aptamer-siRNA conjugates subsequently fuse with early endosomes which mature into late endosomes, and finally enter into the lysosomes that are the last compartment of the endocytic pathway.^[70] It is known that the acidic lysosomal environment (approx. pH 4.8) contains a high content of lysosomal membrane proteins and active lysosomal hydrolases. Therefore, lysosomes can break down cellular products, fats, carbohydrates, proteins, and other macromolecules (such as nanocarriers, RNA, DNA) into simple constituents.^[71] Currently, the mechanism of endosomal release of the internalized aptamer-siRNA conjugates is poorly understood. After endosomal escape, the siRNA portion is processed by Dicer or directly enters into RISC triggering target mRNA degradation. In this regard, some optimization for aptamer-siRNA conjugates should be considered to facilitate endosomal escape and enhance Dicer processing and RISC entry.

4.1 Aptamer-Small Interfering RNA (siRNA) Chimeras

We recently adopted an approach that combines the inhibitory functions of an anti-HIV-1 gp120 aptamer with that of an siRNA targeting an HIV-1 *tat/rev* common exon.^[15,28] This dual action construct has been demonstrated to be effective *in vitro* as well as in an HIV-1-infected humanized animal model in selectively targeting HIV-1 gp120-expressing infected cells and effectively suppressing HIV-1 replication. As shown in figure 1a, the 2'-F modified anti-HIV-1 gp120 RNA aptamer was covalently fused to the sense strand of an anti-HIV *tat/rev* siRNA portion that was annealed with an unmodified antisense strand. A 2~4-nucleotide linker was added between the 3'-end of the aptamer and the siRNA portion to increase the flexibility of the chimera and allow Dicer processing. The resulting chimeric RNA molecule was selectively internalized into cells expressing HIV-1 gp120, and specifically inhibited expression of the targeted mRNA (figure 2). Interestingly, compared with a traditional 21-mer siRNA, a 27-mer Dicer substrate siRNA linked to the 3'-end of the gp120 aptamer provided better silencing (~20% improvement). This might be attributed to the fact that Dicer-generated 21–23 nt siRNAs are more efficiently incorporated into RISC through physical association of Dicer with the Argonaute proteins.^[72,73] In our subsequent study,^[30] we further evaluated the efficacy of this gp120 aptamer-siRNA chimera administrated intravenously to HIV-1 infected, viremic, humanized RAG-humice. Our results showed that treatment of these animals with this chimeric molecule resulted in several orders of magnitude reduction in viral loads and was effective in preventing CD4 T-cell depletion. As expected, the anti-gp120 aptamer itself inhibited HIV-1 infection, while this aptamer-siRNA chimera provided an enhanced duration of inhibition and delivered functional siRNA into the HIV-infected cells as validated by 5'-RACE (rapid amplification of cDNA ends) PCR on the target mRNA sequences. Specific knockdown of targeted mRNAs and siRNA expression were only detectable in the gp120 aptamer-siRNA chimera-treated mice and not in mutant aptamer-siRNA chimera- or naked siRNA-treated animals. Taken together, our results validated the dual inhibitory function of the aptamer-siRNA chimera *in vivo* and provided a strong rationale for clinical testing of aptamer-siRNA chimeras in HIV-infected patients who cannot tolerate the current anti-HIV drugs or who have developed resistance to anti-retroviral drugs.

CD4-targeting RNA aptamers have been shown to block HIV-1 and CD4 interactions and have been proven to be useful anti-HIV agents. Using the same aptamer-siRNA fusion approach,^[74] an RNA aptamer specific for human CD4 receptor was also covalently joined to 21-mer siRNAs targeting the CCR5 coreceptor, HIV-1 *gag* or *vif* genes and triggered specific gene silencing in cultured CD4⁺ T cells and macrophages. Furthermore, the CD4 aptamer-siRNA chimeras (figure 1) were tested in human cervicovaginal tissue explants. When the chimeras were intravaginally administrated to humanized mice, HIV vaginal transmissions to cervicovaginal explants and to the mice were significantly prevented. Different from the work with gp120 aptamer-directed siRNA chimera described above, the CD4 aptamer-siRNA chimeras were applied topically, which could reduce the amounts needed for efficacious treatment compared with systemic delivery.

4.2 Aptamer-Stick-siRNA Conjugates

As mentioned above, the rapid emergency of viral escape mutants often abrogates RNAi activity. Therefore, we also developed a non-covalent 'sticky bridge' approach for conjugating various anti-HIV siRNAs to a single gp120 aptamer.^[29] In this design format (figure 1b), one pair of complementary guanosine and cytosine (GC)-rich 'sticky bridge' sequences was chemically appended to the 3' end of the aptamer and one of the siRNA strands, respectively. Both the aptamer and siRNA portions are chemically synthesized and subsequently annealed via 'sticky bridge'. A flexible three-carbon atom hinge was added as a linker between the 'sticky bridge' and the aptamer to allow spatial and structural

flexibility, as well as between the 'sticky bridge' and the siRNA portion to allow Dicer entry and processing, respectively. In our study, one gp120 aptamer was complexed independently with three different 27-mer Dicer substrate siRNAs, which targeted HIV-1 *tat/rev* gene and HIV host dependency factors CD4 and TNPO3 (transportin-3). The resulting aptamer-stick-siRNA cocktail showed specific binding and internalization into gp120 expressing cells. The conjugates not only efficiently down regulated targeted expression and suppressed HIV-1 replication in HIV-1-infected human leukemic CEM cells and peripheral blood mononuclear cells, but also served as dual function inhibitors which provided additive efficacy. Using a humanized Rag^{2(-/-)gammac(-/-)} (Rag-hu) animal model, a reduction of HIV-1 infectivity was observed, particularly when the three siRNAs were used in the cocktail format (Zhou et al., unpublished data).

4.3 The Aptamer-siRNA Chimera versus Aptamer-Stick-siRNA Conjugate

As described in sections 4.1 and 4.2, we developed and tested two different types of dual inhibitory function anti-gp120 aptamer-siRNA conjugates. For the aptamer-siRNA chimera, both the aptamer and one strand of the siRNA are covalently joined, and synthesized via *in vitro* T7 transcription reaction. In the aptamer-stick-siRNA conjugate, the aptamer and the siRNA are non-covalently assembled through one pair of complementary GC-rich 'sticky bridge' sequences. In our experimental systems (cell culture and humanized mouse model), the aptamer-stick-siRNA conjugate showed comparable anti-viral activity to the aptamer-siRNA chimera.

Currently, the RNA chemical synthesis technology limits the length of the RNAs. Even though the longer (100 nt) modified RNA can be obtained through *in vitro* transcription, the RNA yield is much lower than that obtained from chemical synthesis. In this regard, the 'sticky bridge' approach offers a notable advantage of chemical synthesis with less cost. Moreover, this unique strategy theoretically can be used to facilitate assembly of different siRNAs with various aptamers, which is necessary to avert viral resistance to the siRNA components.

5. Conclusion and Perspectives

So far, in humanized mice models, cell-specific aptamer-mediated siRNA delivery systems for the treatment of HIV-1 infection work very well. Despite these successes in HIV therapy, the aptamer-siRNA conjugates must be further optimized and refined for clinical translation. For example, it is necessary to increase pharmacokinetic profiles with prolonged circulation and effective biodistribution to treat the various reservoirs of HIV infection such as the gastrointestinal lymphatic system. Meanwhile, the kinetics of aptamer-siRNA conjugate uptake and the mechanism of endosomal release still need to be investigated and fully understood. Currently, aptamer-mediated siRNA therapeutics are limited by the number of cell-specific aptamers available. To validate their safety and efficacy testing in non-human primates would be advantageous prior to human clinical applications. Therefore, future efforts should be made to develop novel aptamers against other cellular receptors as well as improve detection methodologies for identifying such cell-specific aptamers.

These examples showing that RNA can be used to deliver siRNAs raise interesting future possibilities for siRNA delivery. Indeed, nucleic acid building blocks consisting of the combinatorial use of aptamers and siRNAs are relatively easy to manufacture. With the technological maturation of RNA chemistry and increasing knowledge of RNAi and aptamers, it would be attractive to partner the two kinds of nucleic acid therapeutics for expanding the options for HIV-1 therapies.

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Fig. 1.

Aptamer-siRNA conjugates for inhibiting HIV. **(a)** Schematic of aptamer-siRNA chimeras. Cell-internalizing aptamers (HIV-1 gp120 or CD4 RNA aptamer) are covalently fused to siRNAs against HIV-1 *tat/rev*, or *gap*, *vif* or CCR5. A 2~4-nucleotides linker connects the aptamer and siRNA. **(b)** Schematic of aptamer-stick-siRNA conjugates. Cell-internalizing aptamer (HIV-1 gp120 aptamer) is non-covalently conjugated to 27-mer Dicer substrates against HIV-1 *tat/rev*, CD4 and TNPO3. A pair of complementary 'sticky bridges' is employed to connect the aptamer and siRNA portions. **siRNA** = small interfering RNA.

Fig. 2.

Aptamer-mediated cell-type-specific siRNA internalization and targeting RNAi. **(a)** Upon binding of the aptamer portion of the aptamer-siRNA conjugates to the target receptor on the cell surface, **(b)** the complex is internalized into cells, probably through a receptor-mediated endocytosis pathway. **(c)** It is presumed that the complex shuttles into the endosome; **(d)** subsequently, the aptamer-siRNA conjugates dissociate from the complex and **(e)** escape the endosome. **(f)** The released aptamer-siRNA conjugate is processed by Dicer. **(g)** The resulting siRNA is incorporated into RISC, **(h)** is recognized by the RNAi machinery and then mediates degradation of the target mRNA. **RISC** = RNA-induced silencing complex; **RNAi** = RNA interference; **siRNA** = small interfering RNA.