Published in final edited form as:

J Investig Med. 2014 October ; 62(7): 914–919. doi:10.1097/JIM.0000000000000103.

Cell-type Specific Aptamer and Aptamer-siRNA Conjugates for Targeted HIV-1 Therapy

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Abstract

Human immunodeficiency virus (HIV) is a virus that causes acquired immunodeficiency syndrome (AIDS), a chronic and incurable disease of the human immune system. As the standard of care for the patients with HIV-1, current highly active antiretroviral treatment (HAART) has been therapeutically effective in the majority of patients; however, it is not curative, and HAART is intolerable due to server side effects. Therefore, nucleic acid-based therapeutics, such as antisense oligonucleotide, ribozyme, mRNA, RNAi-based therapeutics, aptamer, etc., have been actively developed as alternative or adjuvant agents for those chemical antiviral drugs in order to surmount those drawbacks. The combinatorial use of various antiviral nucleic acids could be more efficacious in blocking viral replication and preventing the emergence of resistant variants. 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. The present review offers a brief overview of the use of cell-type specific aptamer and aptamer-siRNA conjugates development in our group for the treatment of HIV-1. Their potentials for targeted delivering RNAi therapeutics (e.g. siRNA) and suppressing HIV-1 replication in vitro and in humanized animal model will be highlighted here.

Keywords

HIV-1 gp120 aptamer; HIV-1; RNA interference; siRNA; cell-type specific siRNA delivery

1. Introduction

Therapeutic strategies designed to treat HIV infection with combinations of antiviral drugs (e.g. HAART) have proven to be the standard approach for delaying the progression to AIDS and reducing the risk of death and disease complication.¹ However, dosing regiments

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for the individuals are difficult and drugs must be taken daily. Drug resistance and serious side-effects remain a concern with some patients not responding to HAART.^{2, 3} Nucleic acid-based therapeutics, such as ribozyme, antisense oligonucleotide, siRNA, aptamer/decoy, mRNA, might represent a potential alternative or an adjuvant to the chemical antiviral agents.^{4, 5}

The early development of nucleic acid-based anti-HIV-1 agents mainly included antisense oligonucleotides (ASOs) and RNA decoys.^{6–8} For instance, ASOs specific to TAR (trans-activating response region), RRE (rev response element), HIV Gag (group-specific antigen) and HIV Env (envelop), and RNA decoys against TAR and RBE (Rev binding element) have been reported for inhibiting HIV-1 replication.^{8–10} Later on, ribozyme and aptamer have rapidly emerged as important HIV-1 suppressors.^{11–14} Currently, a number of ribozymes or highly specific aptamers targeting various HIV-1 genomes and protein, or HDFs (HIV-1 dependency factors), including HIV-1 Gag, TAR, Vif (Viral infectivity factor), LTR (long terminal repeat), RT (reverse transcriptase), IN (integrase), NC (nucleocapsid), Env (gp120 protein), Tat (Trans-Activator of Transcription), Rev (Regulator of Expression of Virion Protein), RNase H, CD4 (cluster of differentiation 4), and CCR5 (C-C chemokine receptor type 5) have been generated and indicated an effective inhibition to viral replication.^{15–19} Most recently, more efficacious RNAi-based therapeutics, such as small interfering RNAs (siRNA) and small hairpin RNAs (shRNAs), have shown unique promise in controlling HIV-1 infection.^{20–22} The entire HIV genome can be specifically targeted by RNAi agents, while some cellular receptors that are essential for viral entry (e.g. CD4, CCR5, CXCR4 (C-X-C chemokine receptor type 4)) or other HDFs (e.g. TNPO3 (Transportin-3), KHDRBS1 (KH domain containing, RNA binding, signal transduction associated 1)) also represent potential targets for siRNA-mediated gene silencing in vitro and in vivo models.^{23–25}

To date, most efficacious drug therapy for HIV uses combination of two or three drugs targeting HIV reverse transcriptase and protease.^{1, 26} It is very likely that combinations can prolong and probably prevent resistant viral variants.^{27–29} Therefore, it should be attractive to develop combinatorial nucleic acid-based therapies, which would be more potential in suppressing viral replication and minimizing the viral mutation escape in comparison to single nucleic acid therapeutic agent. Analogous to HAART, four shRNAs were incorporated in a single lentiviral vector, which prevented HIV-1 escape in an in vitro cell culture system.³⁰ In addition, a triple combination of an anti-HIV-1 Tat/rev shRNA with a nucleolar-localizing TAR RNA decoy and an anti-CCR5 hammerhead ribozyme has been constructed (lentiviral vector rHIV7-shI-TAR-CCR5RZ) to effectively combat HIV-1 replication in primary hematopoietic stem cells (HSCs).³¹ Importantly, a prolonged viral suppression over 6 weeks was observed, thus providing more effectiveness than an anti-HIV-1 Tat/rev shRNA alone or double combinations of the shRNA with ribozyme or shRNA with decoy. Recently, the triple combinatorial construct has been evaluated in a human clinical trial (ClinicalTrials.gov identifier NCT00569985).³²

The technical term “aptamer” came from Latin word *aptus* “to fit” and the Greek word *meros* for “part”. Nucleic acid aptamers are *in vitro* evolved single-stranded DNAs or RNAs that can fold into a specific three-dimensional conformation to fit together with their targets.

With the development over the past 24 years, numerous nucleic acid aptamers have been evolved against thousands of various targets, including small metal ions, organic dye molecules, short peptides, large proteins, viruses, and even whole cells or organisms. Many research and review articles have described their selection and application in the different fields. Due to their nature of nucleic acid, the DNA or RNA aptamers can form specific, diverse structures, such as stem, internal loop, hairpin, bulge, pseudoknot, G-quadruplex motif or kissing complex, which are essential for the target recognition. Due to the exquisite specificity and binding affinity, nucleic acid aptamer itself can stand alone as a potential therapeutics.^{33, 34} In 2005 the first therapeutic aptamer, called Pegaptanib (Macugen) offered by OSI Pharmaceuticals, a PEG-modified RNA molecule, has been approved by FDA for the treatment of age-related macular degeneration (AMD). Additionally, at least ten aptamer-based therapeutic agents are currently being evaluated in the clinic phase I and II.

On the other hand, cell-type specific aptamers, as a building block, can be assembled with other therapeutics or delivery system, thus resulting in the specific recognition, and further improving cellular internalization of the delivered cargoes.^{35, 36} Various molecules or delivery vehicles, such as anticancer drugs, toxin, enzymes, radionuclides, virus, antisense oligonucleotides, RNAi triggers (siRNAs, shRNAs, microRNAs), and nanomaterials, have been functionalized by cell-type specific aptamers for targeted delivery purpose.^{35, 37} Recently, several groups have successfully adapted HIV-1 gp120-specific aptamers^{38, 39} and host cellular receptor CD4 aptamers^{40, 41} for targeted delivery of siRNA molecules into cultured cells and humanized mice model. Our laboratory is interested in developing and testing complementary highly innovative strategies to target HIV-1 infected cells, and specifically regulate both gene silencing of HIV-1 and inhibition of the CD4 receptor-HIV-1 envelope glycoprotein (gp120) interaction required for HIV-1 to enter cells. Through “Purified Protein-based SELEX”, we successfully identified 2'-Fluoropyrimidine modified RNA aptamers from a RNA library that bind to the HIV-1 gp120 with nanometer affinity.³⁹ The selected anti-HIV gp120-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. Our efforts demonstrate that the nucleic acid drugs can be successfully used in combinations resulting in efficacious treatments for HIV infection. The importance of such a strategy for the treatment of HIV-1 cannot be overstated as viral variants resistant to current therapies are on the rise in developed nations and portend a new pandemic of resistant virus. In the following section, we will mainly focus on our own efforts in the development of cell-type specific aptamer and combinatorial use of aptamer and siRNA for HIV-1 therapy. We will highlight representative examples and also discuss the challenges of advancing combination of antiviral aptamer and siRNA therapeutics.

2. Development of HIV-1 gp120 aptamers as HIV-1 inhibitor

SELEX (Systematic evolution of ligands by exponential enrichment) that is an evolutionary strategy to generate high-affinity nucleic acid aptamers was described primarily in 1990.^{42–44} The SELEX condition can be further refined and modified to drive the selection to aptamers with desired features. Currently, two typical SELEX strategies have been

employed to select and identify cell-type specific, nucleic acid aptamers. One is to use traditional purified membrane protein as target,⁴⁵ while another is to use whole live cells as selection target.^{46, 47} Recently, some variants also have been developed.⁴⁸ For example, alternate use of these two procedures has been reported to improve the specificity to the target protein. Apart from whole cells, live animal / organ has also been used as target for SELEX, which is so-called “in vivo SELEX”.^{49, 50}

HIV external envelope gp120 glycoprotein that is exposed on the surface of viral particles and the plasma membrane of HIV infected cells represents a biomarker to distinguish HIV-1 infected cells from non-infected cells.^{51, 52} The interaction between the HIV gp120 and the human cellular CD4 receptor is a crucial trigger in the entry of HIV into T-cells, subsequently resulting in fusion of the viral membrane with the host cell membrane.^{53, 54} Therefore, generating some blockers directed to the virus surface gp120 protein should be a direct and rational antiviral strategy to prevent the viral infection.⁵⁵ Several success examples in development of nucleic acid aptamers against HIV-1 gp120 that are capable of blocking the interaction of gp120 and CD4 have been reported in recent years.^{56–60} For example, William James and co-workers have described the selection and characterization of several anti-HIV gp120 RNA aptamers using a BIAcore biosensor system. They demonstrated that the selected aptamer specifically bound to the conserved co-receptor interacting region of an R5 HIV-1 strain and suppressed HIV-1 infectivity in human peripheral blood mononuclear cells (PBMCs).⁵⁷

Our own efforts have also added several novel, RNase resistant RNA members in the family of anti-HIV gp120 aptamer.⁶¹ We employed a standard in vitro SELEX procedure, in which a purified HIV-1_{BaL} gp120 envelop protein (R5 strain) as target and an RNA library containing 50 random nucleotides was used for the selection. The RNA/protein complexes were isolated from the unbound RNA species through a nitrocellulose filter-based partition method. Subsequently, the bound RNAs were recovered from the nitrocellulose membrane for the cDNA synthesis and re-amplification. New transcripts of the DNA products were applied for the next round of selection. After twelve rounds of selection, the individual aptamer sequences were identified via cloning and sequencing analysis. A-1, one of best aptamer candidates, displayed good binding kinetics to the target gp120 protein with low nanomolar dissociation constant (52 nM of apparent K_d value) calculated from a native gel mobility shift assay. Our results demonstrated that A-1 aptamer specifically bound, and then was rapidly internalized into the cells expressing HIV-1 gp120 protein. Importantly, A-1 aptamer significantly neutralized the replication of various HIV-1 strains (R5- and X4-tropic laboratory and clinical HIV isolates) in cultured CEM T-lymphocytes and in human PBMCs, suggesting the anti-HIV-1 gp120 aptamers offer potential, lead anti-HIV-1 inhibitors for the treatment of HIV-1.

3. The strategies of aptamer-siRNA targeting in HIV therapy

Because the selective binding of anti-HIV-1 gp120 aptamers to the cells expressing HIV-1 gp120 protein can trigger rapid cellular internalization, the gp120 aptamers probably had the potential to be a cell-type specific, internalizing targeting molecule for selective drug delivery to HIV-1 infected cells. We therefore took advantage of this property to explore

aptamer-targeted RNAi strategies. It is expected that the combinatorial use of two nucleic acid therapeutics (gp120 aptamer and siRNA) would offer a “dual-inhibitory function” bonus for combating HIV-1. Our laboratory has developed two different types of anti-gp120 aptamer-siRNA conjugated (Figure 1). The design and activity will be discussed in the following paragraphs.

3.1 Covalent aptamer-siRNA chimeras

By adopting a similar strategy as previously described by Giangrande et al.,⁶² we covalently fused an anti-HIV gp120 aptamer (A-1) to a Dicer substrate siRNA (DsiRNA) targeting HIV-1 *tat/rev* common exon.^{38, 61} In this design (Figure 1A), the 2'-Fluoro modified gp120 aptamer A-1 covalently joined to the sense strand of *tat/rev* DsiRNA portion was generated via in vitro transcription and subsequently was annealed with an unmodified antisense strand to make the chimeric RNA molecule (A-1-*tat/rev* siRNA chimera). In order to improve the molecular flexibility and maintain the correct folding of aptamer, a two nucleotide spacer (UU) was inserted between the aptamer and DsiRNA. Flow cytometric analysis and confocal microscopy study revealed that the chimera maintained the selective binding affinity and internalization efficiency as the aptamers alone. Moreover, anti-HIV efficacy of the resultant chimeric RNA was evaluated in the HIV-1 infected CEM T-cells and PBMCs as described above. We demonstrated that the gp120 aptamer-siRNA chimera was specifically taken up by HIV-1 infected cells and the siRNA portion processed by Dicer was functioning along with the aptamer. HIV-1 p24 ELISA assay indicated that both of the aptamer and chimera neutralized the production of HIV-1 p24 antigen. However, the chimera treatment indeed was the most efficient. Furthermore, significant silencing of the target HIV-1 *tat/rev* gene was only observed in the infected cells treated with the chimera, but not the aptamer alone or the irrelative siRNA delivered by the same aptamer. These results supported that the aptamer delivered intracellularly siRNA, which subsequently triggered RNAi activity.

Encouraged by these cell culture results with aptamer-siRNA chimeras, we moved forward with the next step to evaluate the anti-HIV efficacy of the chimeras in a HIV-1 infected humanized Rag2^{-/-} γ c^{-/-} mouse model (Rag-hu).⁶³ It has been demonstrated previously that HIV-1 infected Rag-hu mice can mirror the main features (e.g. HIV-1 replication and CD4⁺ T cell depletion) of human HIV-1 infected patients, therefore providing an excellent model for the evaluation of anti-HIV-1 agents.⁶⁴⁻⁶⁷ In our study, about three weeks after HIV-1 infection with NL4-3 virus, the Rag-hu mice established infection became viremic. These animals were given five weekly injections of 0.25 nmol of gp120 aptamer A-1 or A-1-*tat/rev* siRNA chimera. The treatment efficacy was evaluated by the plasma viral loads and CD4⁺ cells counts. Our results indicated that the aptamer A-1 or chimeras strongly inhibited HIV-1 infectivity by three logs of magnitude and protected the viral-induced CD4⁺ T cells depletion. In contrast, naked *tat/rev* siRNAs or mutant gp120 aptamer-siRNA chimers did not prevent viral replication and CD4⁺ T cells decline. We also confirmed that the specific delivery and RNAi efficacy of siRNA in PBMCs of these mice with Taqman PCR and qRT-PCR. Our results showed that the *tat/rev* siRNA was detectable in the mice treated by the gp120 aptamer-siRNA chimeras and a 75% to 90% knockdown in the levels of *tat/rev* gene was observed at both the first and third weeks of treatment. When compared with the gp120

aptamer alone, the combination of the aptamer and siRNA offered more significant suppression and prolonged inhibitory effect by several weeks beyond the last injected dose. Taken the results from 5'-RACE [rapid amplification of complementary DNA (cDNA) ends] together, all of these data clearly indicated that the siRNA was specifically delivered by gp120 aptamer and triggered sequence specific degradation of the target mRNA in vivo animal model.

3.2 Non-covalent aptamer-siRNA conjugation via a “sticky” bridge

Ideally, a combinatorial use of various antiviral nucleic acids (e.g., aptamer and siRNA) would be capable of easily combining different aptamers or siRNAs to minimize the viral escape mutants, which often abrogate RNAi activity. To further improve the facility of conjugation, continued efforts have also been made by our group. Different from the aforementioned covalent conjugation in aptamer-siRNA chimera, a “sticky bridge” strategy has been designed to non-covalently assemble aptamer with different siRNAs.³⁹ In this strategy (Figure 1B), the 3'-end of aptamer and one strand of siRNAs was chemically linked to one pair of 16-nt “sticky bridge” sequences, respectively. Since they are highly GC-rich and complementary, a simple incubation at 37 °C for 15 min allows the formation of a stable base-paired conjugate. A flexible three-carbon atom hinge (C3) as a linker was added between the aptamer and sticky sequence to make molecular flexible, thus allowing the correct structure of aptamers and the siRNA processing by Dicer. Currently, commercial solid-phase using phosphoramidite generally makes RNA sequences of less than 60 nt. It is a big challenge to chemically synthesize longer RNA (more than 80 nt) with various modifications, such as 2'-Fluor, 2'-methyl, 2'-amino, etc., via efficient, cost-effect manufacturing. In the “sticky bridge” strategy, all the RNA components as individual building blocks can be chemically synthesized with desired modifications. In the comparison to the longer aptamer-siRNA chimera (> 100 nt), it offers two notable advantages: 1) large-scale chemical production with a relative lower cost; and 2) facile assembly of various nucleic acids components, which may mitigate viral resistance to the aptamer or siRNA therapeutics.

In our study, this strategy facilitated the ready exchange of three different 27-mer Dicer substrate siRNAs, which targeted to HIV-1 tat/rev gene and HDFs (CD4 and TNPO3), associated with one single gp120 aptamer (A-1). The resultant gp120 aptamer A-1-stick-cocktailed siRNA conjugates showed selective binding and cellular uptake to gp120 positive cells, and effectively inhibited viral replication in HIV-1 infected cells. Furthermore, using a HIV-1 infected humanized Rag-hu animal model, we validated the functional delivery of multiplexed siRNAs in vivo mediated by the gp120 aptamer via the “sticky bridge”.⁶⁸ As described previously in the case of aptamer-siRNA chimera, the plasma viral loads were assessed to determine if the established viral loads could be suppressed by the treatment of aptamer-stick-cocktailed DsiRNA conjugates on a weekly basis (5 weekly injections of 0.25 nmol per injection). Compared to the control groups, an average three logs of suppression was observed with a statistical significance. When the treatment was terminated, HIV-1 level was rebounded in the majority of the treated animals, which facilitated a follow-up injection with the aptamer-stick-cocktailed siRNAs conjugates. This follow-up treatment provided complete suppression of HIV-1 levels, which persisted for 2 weeks beyond the

period of follow-up treatment. In the mice treated with the conjugates, the levels of CD4⁺ T cells remained near the levels of the uninfected mice. Moreover, substantial reduction of the three targeted mRNAs was observed in the PBMCs from the treated mice. Collectively, the “sticky bridge” strategy suggested a facile, combinatorial use of antiviral nucleic acids for targeted HIV-1 therapy.

4. Conclusions and challenges

Nucleic acid-based therapeutics has captured the extensive attention of researchers for a long time. The combinatorial use of two or three antiviral nucleic acids has been proven to be more efficacious than single nucleic acid component in the cultured cells and in humanized mice model, even in HIV-1 patients. In our laboratory, we have successfully capitalized on the exquisite specificity of HIV-1 cells-specific aptamers against gp120 to deliver anti-HIV siRNAs in vitro and in vivo, which resulted in additive antiviral activity by the combined action of the aptamer and siRNA. The gp120 aptamers can function both as HIV-1 entry inhibitor and as targeted delivery agents for siRNA.

Despite of these successful examples, several key challenges hinder the rapid translation of aptamer-mediated therapeutics to clinic. Long-term and repeated administration of nucleic acid therapeutic may cause some potential toxicities, including non-specific immune response, polyanionic effects, tissue accumulation, and off-target effect, etc. Additionally, the pharmacokinetics (PK) of nucleic acids therapeutics should be taken into account.^{69, 70} Due to the relatively smaller size and molecular weight (40–60 nt, 15–20 kDa), nucleic acid aptamers or siRNAs are vulnerable to nuclease degradation, and are subjected to rapid renal filtration and short circulation half-time in the blood organisms. Therefore, rational modifications should be considered to improve their PK profile for clinical development.^{33, 71} For example, we incorporate 2'-Fluoro protective group in the gp120 RNA aptamers, thus significantly enhancing their nuclease resistance in mouse serum and in animal model.

Moreover, effective endosomal release of the aptamer-mediated siRNA delivery system still remains an important concern. A higher concentration of aptamer-siRNA conjugates was required to obtain effective gene silencing. Although efficient cellular internalization of large amounts of conjugates was observed, siRNA-mediated knockdown was marginal. The majority of siRNA may be trapped in the endosome and be inaccessible by Dicer and RISC (RNA-induced silencing complex) in the cytoplasm. Therefore, further efforts should be necessary. For example, formulating multiple aptamers and siRNAs into nanocarriers with endosome rupturing capability may increase targeting capability of aptamers and loading capacity of siRNA. Advances in chemical synthesis and nanotechnology would hopefully solve these concerns in the near future.

Acknowledgments

Funding: This work was supported by National Institutes of Health grants R01AI29329, R01AI42552, and R01HL07470 to J.J.R., and P01AI099783 to Kevin Morris (The Scripps Research Institute) and subcontracted to J.J.R.

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Figure 1. HIV-1 gp120 aptamer-mediated siRNA delivery system. (A) Schematic of the covalent aptamer-siRNA chimera. The chimera was synthesized as two pieces followed by an annealing step to make the chimera. A two nucleotide (UU) overhang (green) was added between the aptamer and sense strand of siRNA. (B) Schematic of the aptamer-stick-siRNA conjugate. The aptamer and siRNA were appended to complementary 17-nt 2' OMe/2' Fl GC-rich bridge sequences and were annealed by simple mixing that allowed Watson-Crick base pairing.