

## REVIEW

# RNA-based Gene Therapy for the Treatment and Prevention of HIV: From Bench to Bedside

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Gene therapy is considered a feasible approach for the treatment and prevention of HIV/AIDS. Targeting both viral genes and host dependency factors can interfere with the viral lifecycle and prevent viral replication. A number of approaches have been taken to target these genes, including ribozymes, aptamers, and RNAi based therapies. A number of these therapies are now beginning to make their way into clinical trials and providing proof of principle that gene therapy is a safe and realistic option for treating HIV. Here, we focus on those therapies that have progressed along the pipeline to preclinical and clinical testing.

## INTRODUCTION

Highly Active Antiretroviral Therapy (HAART†) has been extremely effective in controlling the disease progression kinetics in HIV-infected individuals. The prohibitive costs of the HAART regimen restricting its widespread use in developing countries, the associated side effects leading to non-compliance, and thus the development of drug-resistant viral strains have

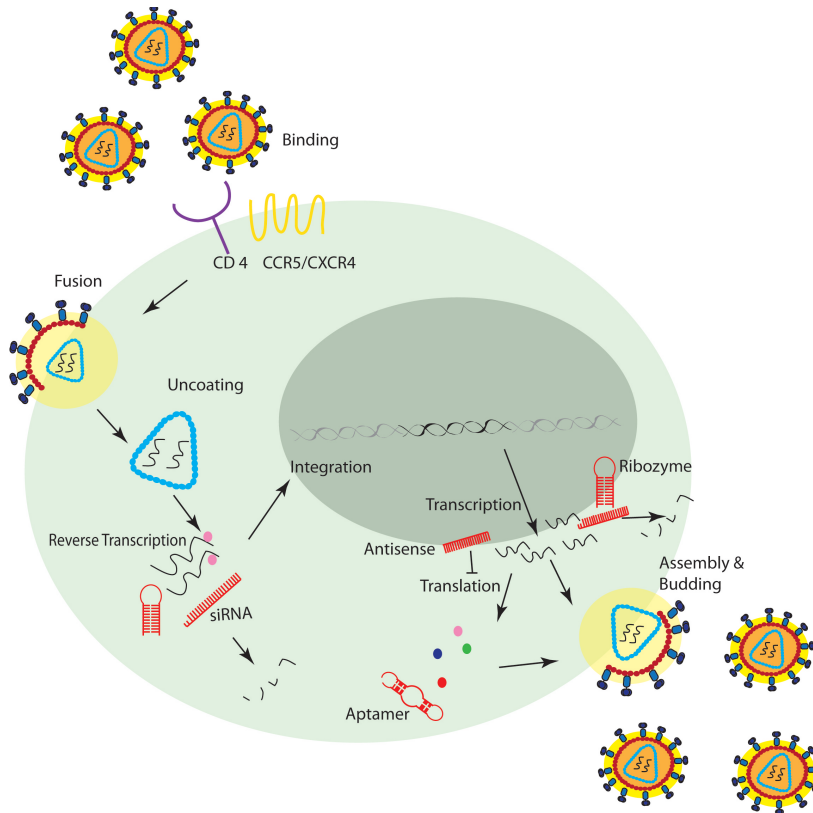
contributed to the current alarming numbers of 34 million HIV-infected individuals worldwide [1]. This underscores the importance of developing an effective approach that can either prevent viral infection upon exposure or transmission from infected individuals. In this review, we will focus on RNA-based genetic therapies that have been evaluated in a clinical setting. These drugs fall into two cate-

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†Abbreviations: HIV, human immunodeficiency virus; HAART, Highly Active Antiretroviral Therapy; RT, reverse transcriptase; PBMC, peripheral blood mononuclear cell; AIDS, Acquired Immune Deficiency Syndrome; RNAi, RNA interference; shRNA, short hairpin RNA; siRNA, small interfering RNA.

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**Figure 1. The lifecycle of HIV.** HIV first binds to the cell via gp120 recognizing CD4 on the host cell. This causes a conformational change that exposes the co-receptor binding loop of gp120 and allows it to bind to co-receptor CCR5 or CXCR4. This causes fusion of the viral envelope with the host cell membrane and leads to viral uncoating and reverse transcription. The DNA is imported into the nucleus and integrated into the host cell's genome, where it can then undergo transcription and translation using host cell machinery. New virions are assembled at the host cell membrane, where they are released by budding. For gene therapy approaches, we can either target host dependency factors utilized by the virus to complete this lifecycle or we can target viral genes. siRNA and ribozymes can target the viral RNA during uncoating and following transcription, leading to degradation of the RNA. Antisense oligonucleotides bind viral RNA after transcription, preventing translation. Aptamers serve as RNA decoys that can bind the viral proteins, preventing them from carrying out their function in the viral lifecycle. Single squiggly lines represent RNA, double helix represents DNA, and circles represent viral proteins.

gories: those that inhibit autonomously such as ribozymes and aptamers or those that rely on cellular machinery to inhibit viral replication such as RNAi-based approaches. Both approaches have advantages and disadvantages. siRNA relies on host machinery to be guided to its target, where ribozymes or aptamers interact with target RNA via diffusion [2]. However, RNAi can saturate endogenous processing machinery or elicit off-target effects leading to dysregulation of host cells.

The HIV lifecycle begins as it infects a new host cell (Figure 1). HIV gp120 binds to CD4 receptor, causing a conformational change in gp120 and allowing binding to a co-receptor, either CCR5 or CXCR4. Binding to a co-receptor causes fusion of the viral envelope with the target cell membrane, resulting in viral uncoating and exposure of the dimeric RNA genome. This genome is then reverse transcribed into double-stranded DNA by viral reverse transcriptase (RT) and imported into the

nucleus, where integration occurs using viral integrase. Replication of the viral genome is divided into two phases, the first of which includes multiply spliced transcripts encoding *rev* and *tat*. These regulatory proteins are then re-imported into the nucleus, where *tat* drives more viral transcription and *rev* escorts singly spliced and unspliced transcripts out of the nucleus to make the proteins of late phase replication. These proteins assemble at the host cell membrane in virions that are released through budding. Once released from the cell, viral protease continues to cleave polyproteins into the mature form, creating infectious virus. This life cycle of HIV is dependent on both viral and cellular factors that when disrupted may impact viral replication. Viral targets present a unique and distinct factor that should have little impact on host cell function. However, the high mutation rate of HIV and the relatively small changes required to escape inhibition may lead to the emergence of escape mutants. Targeting the more immutable cellular factors (e.g., CCR5) provides a lower chance of mutation, but long-term inhibition of cellular factors may lead to deleterious effects in host cells. Presented in this review are the approaches that have been taken to target these genes in clinical studies by RNA-based genetic therapies, including ribozymes, RNAi, and aptamers.

## RIBOZYMES

Ribozymes are small, catalytically active RNA molecules, the most well studied being the hammerhead and the hairpin ribozymes. The hammerhead ribozyme is one of the smallest ribozymes and is composed of 30 nucleotides with a conserved core and three stems [3]. Natural hammerhead ribozymes are self-cleaving molecules. However, these ribozymes can be engineered for *trans* cleavage. These *trans*-cleaving ribozymes are composed of a conserved catalytic domain, a structural domain, and a target binding domain that provides the sequence specificity [4]. The only requirement for the target domain is a 3-nucleotide con-

sensus sequence, thus allowing the flexibility to target a wide range of genes [4]. The hairpin ribozyme is slightly larger than the hammerhead (50-60 nucleotides), yet cleaves substrates at more physiologically relevant Mg<sup>2+</sup> concentrations than the hammerhead [5]. Hairpin ribozymes are derived from the minus strand of the tobacco ringspot virus satellite RNA. Like the hammerhead ribozyme, hairpin ribozymes are naturally self-cleaving but can be engineered to cleave specific sequences by mutation of the substrate recognition sequences flanking the cleavage site sequence [5]. *Trans*-cleaving ribozymes are of great therapeutic interest as they can cleave multiple copies of substrate in a sequence-specific manner, thus removing genes of interest from the genome.

The first clinical study to use ribozymes to target a disease was conducted in HIV positive patients in 1996 [6]. Hairpin ribozymes were developed to target HIV U5 [7] and *pol* [8] and contain the stem loop II sequence of HIV-*Rev* [9], thus serving as both a ribozyme and a RNA decoy. These constructs protected Jurkat T cell line and PBMC from HIV infection with both laboratory strains and clinical isolates [10,11]. Given the *in vitro* success of ribozyme therapy, a clinical trial was launched to test the safety and feasibility of a ribozyme-based approach for HIV therapy [6]. PBMCs were transduced *ex vivo* with a retroviral vector pMJT expressing two hairpin ribozymes directed against HIV U5 and *pol* and reinfused into three HIV-positive subjects. Achieving high transduction efficiency and reconstitution with these cells *in vivo* proved to be challenging despite the promising *in vitro* data. Vector was detected in only one patient post-infusion, and no detection of the MLV-driven *pol* ribozyme was achieved [6,12]. However, in this patient, the U5 ribozyme driven by tRNA<sup>Val</sup> promoter was detected at 5 and 7 months post infusion, suggesting that this promoter is better suited for *in vivo* ribozyme expression. Despite low efficacy of vector, this study marked the first clinical trial using ribozymes and demonstrated safety of ribozyme transduction into PBMC.

To study the safety and feasibility of ribozyme delivery to CD34+ cells, murine retroviral vectors were created to deliver hammerhead ribozymes targeting HIV *tat* and *rev* driven from the vector LTR [13]. These vectors effectively transduced CD34+ cells and showed a 1,000-fold inhibition of HIV replication *in vitro* [14]. Two phase I clinical trials were initiated utilizing these vectors for *ex vivo* transduction of CD34+ cells in HIV positive patients showing no symptoms or HIV positive lymphoma patients [15,16]. For the first study, no myeloablation was performed and presence of vector was detected in only three of five patients, persisting in one patient for up to 6 months [15,16]. However, with myeloablation in a bone marrow transplant setting, four of five patients showed high levels of marking initially and persistent marking throughout 1 year. A higher proportion of PBMC were marked with vector carrying the two ribozymes than the control vector, suggesting a selective advantage was conferred to these cells, and RNA expression was detected in PBMC of these four patients. While these results are promising, the levels of expression sharply declined, becoming undetectable after 1 year. This provides evidence that while transduction of CD34+ cells with ribozymes is a feasible approach, improvements in transduction efficiency must first be established.

Johnson and Johnson Research also has developed a ribozyme targeting HIV *tat* and has now tested this therapy in the first phase II cell-delivered clinical trial for HIV [17]. A hammerhead ribozyme (Rz2) targeting the overlapping *vpr* and *tat* reading frames of HIV-1 was developed and tested in T cell lines, primary T cells, and macrophages [18-20]. Rz2 demonstrated high levels of inhibition of replication of laboratory strains and AZT- and nevirapine-resistant HIV. No emergence of escape mutants was observed, and non-cleaving ribozymes showed far less inhibition than catalytically active ribozyme [19]. Retroviral transduction of CD34+ cells with Rz2 showed high transduction efficiency and had no deleterious effects on differentiation of CD34+ in SCID-hu mice [21].

This vector was clinically tested in two concomitant phase I trials transducing either CD4+ T cells [22,23] or CD34+ cells [21,24]. To test transduction efficiency in CD4+ T cells, four pairs of HIV discordant identical twins were enrolled in a phase I trial. HIV negative twins served as donors, and CD4+ cells were isolated, transduced with either Rz2 or control vector (LNL6), expanded, and infused in HIV positive recipient twin [22,23]. In all patients, marking and expression of ribozyme was observed at all time points through at least 2 years and again in the three patients that returned for a 6-year time point [23]. Sequencing of quasispecies isolated from these patients showed that no mutations had occurred within the cleavage site [23]. This study showed that expression of Rz2 is highly stable in CD4+ lymphocytes and transduction of these cells has no deleterious effects on survival.

Similar results were seen when CD34+ cells were transduced with Rz2 and autologously reinfused [21,24]. Ten HIV-positive patients were recruited for this study. Transduction efficiencies for the first three patients were low (< 1 percent to 4 percent) and marking was seen up to 12 months [24]. The next seven patients were transduced in the presence of CH296 fibronectin, increasing transduction efficiencies to 32 percent. Transgenes were detected in all patients at multiple time points during the follow-up with the latest positive detection at 36 months. CD4+ T cell counts increased over the 3-year period and viral load decreased by an average 2.25 logs in six patients. As with the CD4+ lymphocyte transduction, no mutations in the catalytic target site were seen. Results from this phase I trial prompted a phase 2, multicenter, double-blind, placebo-controlled clinical trial to assess feasibility and safety of this gene therapy approach [25]. Seventy-four early-stage HIV-positive patients were divided into OZ1 (formerly Rz2) or placebo infused into CD34+ cells. All patients' viral load was suppressed by HAART, and two HAART treatment interruptions were conducted on weeks 24 to 28 and again at weeks 40 to 48. The second treatment interruption was al-

lowed to continue until the completion of the study, week 100, as long as the patient met study parameters. Initially, marking was observed in 94 percent of OZ1-infused patients but declined by week 100 to 7 percent. By week 100, 12 participants remained on HAART treatment interruption and OZ1 RNA was detected in five of these subjects. The mean plasma viral loads for the OZ1-treated group were lower, yet not significantly. However, in a subset of patients that continued to express OZ1 RNA, a statistically significant decrease in viral load was observed. While the low engraftment levels and short persistence of the ribozyme are disheartening, it is encouraging that efficacy was seen in patients that did express OZ1 RNA and that infusion was well tolerated, causing no apparent adverse events.

The clinical trials described so far have focused on the use of ribozymes alone. However, the combination of ribozymes with additional RNA-based strategies may help prevent escape mutants and attack the virus with multiple approaches. Recently, this concept was tested in a phase I clinical trial combining a CCR5 targeting ribozyme (R5RZ), a TAR decoy, and shRNA targeting a *tat/rev* exon (shI) [26]. *In vitro* studies first focused on characterizing the individual components of this vector [27-29] and then the double vector with TAR decoy and R5RZ. Both T cell lines and CD34+ cells were protected against HIV infection and provided a selective advantage over untransduced cells [29,30]. Combination of the TAR decoy, the R5RZ, and shI showed a 4-log reduction in HIV titers and provided stable transduction of CD34+ without affecting characteristics of differentiated macrophages [30]. These cells also differentiated similar to controls when used to reconstitute SCID-hu mice [31]. Clinical trials were initiated with four AIDS lymphoma patients receiving bone marrow transplant of both vector transduced and untransduced CD34+ cells [26]. Patients remained on HAART throughout the duration of the study and had undetectable viral loads. Encouragingly, all patients remained in remission from leukemia and had marking of PBMC including T cells, monocytes, and

B cells up to 24 months following infusion. A second phase 1 trial is currently recruiting patients that have failed anti-retroviral therapy for a multi-dose study with infusion of transduced T cells [32].

While none of these trials alone have proved therapeutic effect, taken together they provide a proof-of-principle that ribozyme therapy could be an efficacious approach to the treatment of HIV. Improvements in transduction efficiencies of CD34+ and CD4+ cells paired with increased engraftment and expansion of these cells *in vivo* could lead to greater therapeutic effects.

## RNAi-BASED STRATEGIES

RNA Interference (RNAi) utilizes short RNAs to mediate degradation of mRNA in a sequence specific manner. RNAi can be delivered either as a synthetic 21 basepair double-stranded RNA (siRNA) or as a stably expressed precursor short hairpin RNA (shRNA). Additionally, long antisense oligonucleotides can be designed to bind to mRNA and trigger degradation of mRNA through a RNase H dependent pathway or block ribosome binding, inhibiting gene expression. During the HIV lifecycle, HIV RNA is exposed to the cytoplasm both initially upon viral uncoating and once transcribed from proviral DNA. This allows ample interaction of potentially therapeutic RNAi-based therapies with viral RNA and should therefore provide an ideal situation to combat HIV.

To our knowledge, to-date clinical studies utilizing RNAi to combat HIV infection have relied on antisense strategies with the exception of the *tat/rev* shRNA, R5RZ, Tar decoy described above [26]. The usage of these long antisense constructs can help prevent escape mutants as large portions of the targeted gene would need to be deleted to allow escape causing severe decreases in fitness [33]. Additionally, antisense that is directed against genes not present in the targeting vector does not compromise vector gene expression and allows for high titer vector production. Therefore, it is not surprising that antisense HIV *env* has been an

attractive therapeutic option [33-37]. Antisense *env* VRX496 was encoded in a conditionally replicating VSV-G pseudotyped lentiviral vector that showed *in vitro* reduction of HIV titers up to 4 logs [35,36]. Phase I trials using VRX496 transduced autologous CD4+ T cell infusion in chronic HIV patients showed high initial engraftment of marked cells with up to  $10^5$  copies/ $10^6$  PBMC [37]. Though this marking rapidly declined with only two of five patients having detectable vector after 2 years, three of five patients showed reduced HIV titers and four of the five showed improvement in CD4+ cell count. Analysis of integration sites showed no preferential integration. Additionally, no replication-competent virus was detected, and only transient mobilization of the vector through day 60 was observed. VIRxSYS Corporation has initiated further clinical testing of this vector, including two phase II trials to further examine dosing and one phase I/II trial withdrawing patients from HAART [38]. While data has not yet been published, 18 patients have currently been enrolled for this phase I/II trial and results to date are said to be encouraging [38].

In addition to antisense *env*, antisense TAR in combination with *trans*-dominant Rev has also been examined in a clinical setting [39]. Ten pairs of HIV discordant identical twins were recruited, and CD4+ cells from the seronegative twin were transduced and infused into the seropositive twin. CD4+ cells were transduced with a single MLV or GALV vector carrying both the dominant negative Rev and the antisense TAR, with vectors carrying dominant Rev alone, or with control vectors carrying no anti-HIV genes. Marking following infusion was between 500 and 1,000 marked cells/ $10^6$  PBMC and declined during the first two months to  $\sim 100$  marked cells/ $10^6$  PBMC. However, though marking decreased over time, the ratio of cells carrying therapeutic vector to control vector shifted 1- to 3-fold, suggesting that a selective advantage was seen in cells carrying anti-HIV therapy. This shift was even more pronounced when one subject was removed from HAART causing

viral rebound, and then infused. Here, the ratio of treatment to control marked cells reached a 100-fold shift, and viral load decreased to half the level at time of infusion.

Enzo Biochem Inc. has also developed and clinically tested its own antisense vector Stealth Vector HGTV43 encoding U1/antisense TAR and antisense directed at two separate regions of the *tat/rev* region [40,41]. In a phase I trial with five HIV seropositive subjects, *ex vivo* transduction of CD34+ and autologous infusion led to marking of CD34+ and CD4+ T cells up to 72 months following infusion. All subjects tolerated the treatment with no serious adverse events, and Enzo has now begun a phase I/II trial to optimize transduction rates [40].

While studies using shRNA and siRNA alone have not yet progressed to clinical trials, a number of promising pre-clinical studies have provided the proof of principle for the use of this technology as an anti-HIV therapeutic. Pre-clinical targets of siRNA treatment have included both host factors such as CCR5 [42-44], viral genes such as *nef* [45], or a combination of viral and host factors [46]. While the current clinical trials rely on *ex vivo* transduction of CD34+ cells or CD4+ cells, preclinical studies using targeted delivery of siRNA or shRNA to HIV susceptible cells give hope for an easy to administer RNAi-based treatment [43,44,46]. Given the recent success of targeted delivery of siRNA in humans for the treatment of melanoma, it will be exciting to see the growth of these strategies in the HIV field [47].

## RNA APTAMERS

Aptamers are single-stranded RNA or DNA molecules that can bind proteins with high affinity serving as a decoy. These molecules, normally 15 to 40 bases long, can be used as decoys to bind viral proteins or as vehicles for targeted delivery of siRNAs. These molecules are being explored for their use *in vivo* as they are non-toxic and non-immunogenic [48]. A recent clinical study discussed above examined the efficacy of a triple construct lentiviral vector expressing a

TAR decoy, R5Rz, and shI [26]. To date, only one clinical trial has used aptamers alone and reported discouraging results [49]. For this study, overexpression of the *rev*-responsive element (RRE) was driven off the LTR of a retroviral vector [14,50]. In culture, this decoy inhibited HIV replication up to 1,000-fold without affecting differentiation of CD34+ cells. However, when four HIV+ children were autologously transplanted with CD34+ cells transduced by MoMuLV RRE decoy vector, marking was only detected in two subjects and disappeared as quickly as 7 days post infusion [49]. Transduction efficiencies of CD34+ cells were between 7 percent and 30 percent and persistence of the control vector LN was seen through 330 days. The low engraftment of these cells could be due to the high rate of quiescent CD34+ cells that are not transducible by MoMuLV and with the improvements seen by using lentiviral based vectors for transduction of quiescent cells, an improvement may be possible.

Preclinically, aptamers to gp120 have been used for their dual function in targeting delivery and inhibiting viral binding [51,52]. Aptamers to gp120 were fused to siRNA targeting *tat/rev* and delivered specifically to CHO-gp160 and HIV-infected CEM T cells without triggering an interferon response [51]. Recently, improvements upon the fused aptamer-siRNA were reported [53]. Here, a GC-rich bridge allows a stable interaction of the aptamer and siRNA, but allows interchange of the siRNA, facilitating the silencing of mutants. These aptamers alone showed HIV inhibition that was further pronounced when “stuck” to anti-*tat/rev*, anti-transportin 3, and anti-CD4 siRNA. Efficacy of these aptamer chimeras were recently tested in the humanized mouse model [52]. Rag2<sup>-/-</sup>γ<sup>-/-</sup> mice were reconstituted with human CD34+ cells and infected with HIV-1 NL4.3 virus. Starting 3 weeks after viral infection, mice were injected weekly with gp120-siRNA chimeras. Decreases in viral load up to three logs of reduction and protection of CD4+ T cells have been reported. The ability of this chimeric aptamer to show efficacy in a therapeutic setting extends hope

for a potential treatment for patients failing HAART therapy.

## CONCLUSIONS

The studies presented in this review provide an overview of the clinical trials that have utilized RNA-based gene therapy for the treatment or prevention of HIV. While none of these studies to date have shown efficacy against HIV, they provide proof of principle that the use of RNA-based therapies is feasible and safe *in vivo*. As advances in vector technology allow for higher levels of transduction of CD34+ and CD4+ cells, the potential efficacy of these therapies can be greatly improved. Many of these studies also rely on a selective advantage for the expansion of transduced cells. This is difficult to achieve in subjects with well-controlled HIV levels remaining on HAART. As these therapies progress along the pipeline, it will be exciting to compare the selective advantages seen *in vitro* to those actually achieved *in vivo*. Additional technological advances in the field have focused on targeted delivery to eliminate the need for expensive and time consuming *ex vivo* transduction. As preclinical studies continue to show the efficacy of these approaches, targeted delivery to T cells may be an achievable milestone in the clinic.

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