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Crippling of HIV at Multiple Stages with Recombinant Adeno-Associated Viral Mediated RNA Interference

Ramesh B. Batchu[1,2] et al.[*]
[1]*Laboratory of Surgical Oncology & Developmental Therapeutics, Department of Surgery, Wayne State University, Detroit, MI*
[2]*John D. Dingell VA Medical Center, Detroit, MI*
USA

1. Introduction

Acquired immunodeficiency syndrome (AIDS) is a disease caused by the infection of human immunodeficiency virus-1 (HIV-1) that primarily impairs immune function by reducing the CD4 T-lymphocyte count. More than two decades after the first clinical evidence of AIDS was reported, AIDS continues to be a major public health problem worldwide with millions of people infected and new infections rising in an alarming rate in third world countries especially in Asia and sub-Saharan Africa.(1, 2) AIDS has become one of the most devastating diseases that the scientific community has ever faced, struggling till today to come up with a therapeutic strategy that successfully controls the disease. AIDS is now the leading cause of death in sub-Saharan Africa, and is presently the fourth biggest killer worldwide. AIDS-related deaths totaled over 5 million by 2009 reaching a cumulative death toll of over 30 million since the beginning of the epidemic. More than 75 million people have been infected with HIV-1, and roughly 2.7 million new HIV-1 infections were diagnosed in 2009(3) even though this rate has decreased from one decade ago.

To date, there is no effective treatment and the number of individuals infected with HIV-1 is growing dramatically in the eastern part of the world. Considering its infection rate, it is imperative to devise newer strategies to control progression of the disease. Although newer approaches such as highly active antiretroviral therapy (HAART) have proven to be effective in prolonging life, other constraints associated with their use underscores the need for development of other effective therapies. Protease inhibitors appear to be successful at controlling the viral replication immediately following budding of immature virus particle, but the development of drug resistant viral mutants and toxicity after prolonged therapy contributes to their failure.(4) HAART has considerable toxicity and its inability to

[*]Oksana V. Gruzdyn[1], Aamer M. Qazi[1,2], Assaad Y. Semaan[1], Shelly M. Seward[1], Christopher P. Steffes[1], David L. Bouwman[1], Donald W. Weaver[1] and Scott A. Gruber[2]
[1]*Laboratory of Surgical Oncology & Developmental Therapeutics, Department of Surgery, Wayne State University, Detroit, MI, USA*
[2]*John D. Dingell VA Medical Center, Detroit, MI, USA*

effectively act on virus in secondary lymphoid tissue is a significant drawback. Vast majority of people with AIDS live in poorer countries. HAART is expensive and unreachable to low and middle-income countries.(5) Many of these places do not have access to HAART, or if they do, supply can be intermittent. The finding that infections with drug-resistant HIV-1 are increasing further underscores the need to develop inhibitors of HIV-1 that are effective, affordable and universally accessible.

With the discovery of RNA interference (RNA*i*) phenomenon, that operates in mammalian cells and is highly effective in selective gene silencing, new, potent, small interfering RNA (siRNA) molecules have become available to add to control HIV-1. By analyzing the challenges of HIV-1 drug development, we review novel and multi-faceted therapies by simultaneously targeting multiple regions of HIV-1 so as to effectively cripple of the virus. The targets include essential cellular genes to avoid viral escape through mutations; multiple regions at various phases of the viral life cycle for a synergistic effect; and anti-sense approach as well to avoid viral escape strategies of HIV-1 against RNA*i*. Current challenges facing the advancement of RNA*i* therapy are its safety and inefficient delivery *in vivo*. Self-complimentary recombinant adeno-associated viral (rAAV-sc) vectors can overcome shortcomings associated with RNA*i*-mediated gene silence therapy.(6) AAV vectors are safe and clinically proven. New generation vectors with mutant capsids circumvent pitfalls of ubiquitin-proteasome mediated degradation leading to high-efficiency transduction at low doses ideally suited to be part of a new arsenal for in vivo RNA*i* delivery to fight HIV-1.(7) Unlike present drugs in the clinical trial or R&D stage, the multi-targeted AAV mediated RNA*i* approach not only kills the virus but also prevents the development of escape strategies and emergence of resistant viruses by simultaneous attack at multiple targets employing multiple technologies.

2. Traditional AIDS therapies

Anti-retroviral drugs such as nucleoside reverse transcriptase inhibitors and non-nucleoside inhibitors are first generation drugs successful in reducing the viral burden.(8-13) Although they prolong life in selected patients, these agents have significant side effects and generate drug resistant viral mutants. Protease inhibitors appear to be most effective at blocking HIV-1 replication, substantially reducing AIDS-related hospital admissions and death rates.(14)

Present-day therapy uses a combination of nucleoside analogues and protease inhibitors known as HAART.(15) HAART has been shown to be effective in controlling the spread of the virus by reducing the plasma viral load to undetectable levels and to some extent depleting the pool of virus in lymphoid tissues. In the past decade, HAART has become more effective with the introduction of several protease inhibitors, but the treatment is expensive and unavailable in poor countries.(5, 15) Approximately 30 million HIV-1 positive people of whom the vast majority live in low- and middle-income countries do not have access to proper treatment. This underscores the need for the development of inexpensive, yet effective drugs that can reach the majority of patients. Despite the apparent success of HAART therapy, the capacity of HIV-1 to establish latent infection of $CD4^+$ T cells allows viral particles to persist in tissues. Some studies indicated that the therapy does not completely eliminate viral replication in secondary lymphoid tissues. HIV-1 was routinely isolated from lymphoid organs of patients even after years of therapy due to continued replication.(16) Moreover, the initiation of HAART even as early as days after the onset of AIDS symptoms, could not prevent the establishment of a pool of latently-infected T lymphocytes.(17)These observations clearly indicate that traditional combinatorial therapies with protease inhibitors and nucleoside

analogs for HIV-1, though effective in selected patients in prolonging life, unfortunately generate drug resistant viral mutants, unacceptable levels of drug toxicity, and are ineffective against virus in secondary lymphoid tissue.

3. Newer approaches to therapy

Introduction of molecules that are able to dominantly interfere with intracellular replication of HIV-1 is known as ''intracellular immunization''. Intracellular immunization by gene therapy strategies offers a promising alternative approach for controlling and managing HIV-1 disease. These include protein-based approaches such as trans-dominant strategies to inhibit HIV-1: toxins, zinc finger nucleases and single-chain antibodies.(18) Protein-based strategies have been the single largest area of anti-HIV-1 gene transfer trials in humans in the recent past.(19) Other RNA-based intracellular immunization approaches include the use of ribozymes and decoys. These second generation ribozymes are RNA molecules that cleave viral transcripts such as *tat*, *rev*, and *gag* at specific sequences targeting HIV-1 at critical stages, and have been shown to reduce HIV-1 levels *in vitro*.(20-22) RNA decoys are RNA homologues, such as TAR and RRE that bind viral proteins and compete with native ligands necessary for replication. They were also shown to inhibit HIV-1 *in vitro*.(20, 23, 24) In comparison with protein-based strategies, RNA-based approaches may have the advantage of not being immunogenic. Both viral or host cellular factors can be targeted, the latter potentially mitigating the possibility of escape mutants, but nevertheless, these trans-dominant approaches had shown initial promise but fell short of practical utility in providing adequate protection. DNA-based vaccines have shown partial success.(23, 25, 26) Anti-sense molecules were shown by several groups to inhibit HIV-1 *in vitro* when targeted towards critical HIV-1 genes such as *tat*, *rev*, and integrase, but the need for large amounts for *in vivo* studies apart from the problems associated with stability contributed to their failure to enter the clinic.(27)

4. RNA interference (RNA*i*): A natural way of gene silencing

Diseases, for which a foreign gene can be identified as the cause, such as in the case of viral infections, are potentially treatable by blocking its expression that will cripple the causative agent. Over the last decade, small non-coding RNA molecules such as short interfering RNA (siRNA), micro RNA (miRNA) and piwi RNA (*pi*RNA), collectively known as RNA interference (RNA*i*), emerged as critical regulators in mammalian gene expression and hold the promise of selectively inhibiting expression of disease-causing genes.(28, 29) RNA*i* is an evolutionarily conserved mechanism of gene inhibition or silencing first described in Caenorhabditis elegans and was shown to produce sequence-specific gene silencing.(30) In 1999, it was recognized as the natural cellular process to destroy unwanted foreign genes such as those causing viral infections(31). In 2001, for the first time, *the use of synthetic siRNA to silence genes in mammalian cells was demonstrated, and was referred to* as 'Biotech's billion dollar break through.(32) In short, RNA*i* has the potential to revolutionize the treatment approach to various diseases. Over the years, it has become clear that RNA*i* is a highly conserved molecular mechanism used by eukaryotic organisms to control gene expression during development and to defend their genomes against invaders, such as transposons and RNA viruses. siRNAs are primarily exogenous in origin, derived directly from the virus or transposon. siRNAs are 21 to 23 double-stranded RNA molecules that recognize the cognate

mRNA with complementary sequence and cleave by naturally occurring cellular mechanisms.(31) *In vivo* silencing occurs after the formation of long double-stranded RNAs that are processed into short interfering RNAs (siRNAs) by an enzyme called Dicer, forming a ribonucleo-protein complex called RNA induced silencing complex (RISC). In the RISC, the anti-sense strand of the siRNA serves as a guide for the degradation of the homologous RNA target. In recent years, siRNA has emerged as a method of choice for specific and efficient gene silencing.

Since discovery of their mechanism, chemically synthesized siRNA molecules are being used to target abnormally elevated genes in many diseases. Since siRNA is a natural biological mechanism against viruses, it can elicit specific intracellular antiviral resistance that may provide a therapeutic strategy against human viruses. siRNAs have been shown to inhibit viral replication or block gene expression in cell culture systems for several viruses. In one study, pre-treatment of cells siRNAs specific to the poliovirus genome promoted the clearance of the virus from most of the infected cells.(33) Shlomai et al observed significant reduction in hepatitis B virus (HBV) by siRNA-producing vectors.(34) A number of groups have demonstrated that siRNAs interfere with hepatitis C virus (HCV) gene expression and replication.(35-39) Over 90% of human cervical cancers are positive for human papilloma virus (HPV) and siRNA-mediated silencing of E6 and E7, the viral genes necessary for the HPV life cycle, completely inhibited them in mammalian cells.(40)

5. Targeting HIV-1 with RNA*i*

Since siRNA can elicit specific knockdown of transcripts and they have been successfully used against human viruses, this ancient defense mechanism can be recruited as a weapon in the fight against HIV-1. Several laboratories have shown that the introduction of siRNAs specific for HIV-1 transcripts has shown viral RNA degradation and inhibition of replication. (41-43) Stable and modified promoters for the expression of siRNA molecules have further shown to increase the potency of HIV *in vitro*.(44) The successful silencing of HIV-1 replication by several investigators through siRNA-mediated targeted knockdown of viral proteins made RNA interference a weapon of choice against HIV-1. (41, 45, 46)

HIV-1 has a total of 15 proteins encoded by 9 genes.(47) Essentially, these can be grouped into four potential target sets for siRNA knockdown. The first potential target for gene silencing is the viral genomic RNA upon viral entry. Jacque et al demonstrated siRNA-mediated destruction of incoming HIV-1(48), although other studies of RNA*i* inhibition of retroviral infection suggested that incoming genomic RNA may not be the best target for siRNAs.(49) Once viral DNA is integrated, the viral mRNA transcripts as well as the un-spliced genomic RNA can be potential target. Early transcripts of HIV-1 such as *rev*, *nef* and *tat* are an important second group of targets for gene silencing with RNA*i* because they not only regulate the subsequent expression of the structural genes, *gag*, *pol*, and *env* but also the synthesis of full length viral genomic RNA. siRNA targeting the *nef* gene has been demonstrated to provide efficient silencing in a transient-transfection system.(50) There have been efficient demonstrations of silencing of the expression of various regulatory genes in a transient-transfection system.(44, 51, 52) siRNAs targeted to the TAR regulatory region and nef of the HIV-1 genome have also been shown to be effective at silencing the level of virus replication and inhibiting reverse transcription intermediates.(53, 54) After regulatory genes, structural genes also represent a potential target group.(55-57) It was found that inhibition was more significant when the siRNA

were present before the viral infection. This is because the vulnerability of genomic HIV RNA for RNA*i*-mediated knockdown is much greater immediately after viral entry into the cytoplasm due to the availability of target transcripts.
A crucial finding was that a high degree of specificity of the RNA*i* for the sequence of its target was required. Even one base pair change dramatically lowers the potency of RNA*i*-mediated inhibition.(58)This becomes important, given the high error rate of HIV-1 reverse transcriptase that leads to the emergence of RNA*i* escape mutants. The HIV-1 virus often becomes resistant to RNAi therapy as a result of the appearance of mutant variants. Because of these mutations, although siRNA directed against various HIV-1 genes shows initial success, the virus may soon escape inhibition within weeks. (44) Silencing evasion can also result from loss of target sequences within viral genomes, owing to the high viral mutation rates. In lymphocytes, for example, the effects of anti-HIV-1 siRNAs were progressively dampened by the emergence of viral quasi species that harbor mutations within the siRNA target sequence.(59) RNA*i*-mediated inhibition with single target has not yet been shown to protect cells against HIV-1 in long-term. RNA*i* could become a realistic therapeutic option, however, if used in a combined fashion while targeting multiple genes to prevent the emergence of mutant viruses. Simultaneous attacks by siRNA on various targets will minimize the escape of the resistant virus.

6. Cellular targets of HIV for RNA*i*

An essential cellular HIV receptor or co-receptor target may have more appeal than viral targets, which are prone to mutations. Cellular mRNAs that encode critical proteins involved in HIV-1 replication may circumvent pitfalls associated with viral escape mechanisms. Targeting cellular genes that are an essential part of the HIV-1 life cycle could therefore be advantageous. CD4 is the primary cellular receptor for HIV-1 entry on T lymphocytes. In addition to the CD4 primary receptor, the cellular chemokine receptors CCR5 and CXCR4, which function as co-receptors for HIV-1, have provided new therapeutic targets and a better understanding of the progression of viral infection. Several investigators targeted cellular proteins necessary for the HIV-1 life cycle by siRNAs and produced decreased levels of virus production.(60, 61) Preliminary observations from various laboratories have demonstrated that siRNAs specific for CD4 receptor do indeed inhibit HIV-1 replication.(62, 63) After transfection of cultured T cells with siRNA against the mRNA for CD4, HIV production, after exposure of the cells to the virus, decreased substantially. (64) CCR5, an HIV-1 co-receptor for the M-tropic HIV-1 variant, providing an attractive cellular target for siRNAs since homozygous deletions of CCR5 effectively confer protection from HIV-1 without any serious deleterious effects in immune function.(65) At least one group has taken advantage of this target for RNA*i*-mediated gene silencing, demonstrating that in vitro knockdown of CCR5 by siRNAs provided marked protection from HIV-1 infection.(63)
Although suppression of the primary receptor CD4 may be restricted by its normal role in the immune system, CCR5 seems dispensable for normal life.(66) Unfortunately, not all HIV-1 strains require CCR5, and the inhibition of CCR5 may result in the selection of HIV-1 variants that use CXCR4 as a co-receptor. It is critical to identify this particular aspect by studying strain variants.

7. Escape strategies of HIV-1 from siRNA

One of the hallmarks of RNA*i* is its sequence-specific knockdown of the target transcript, but unfortunately, it also presents a way out for HIV-1, since single nucleotide substitutions

in the target region can drastically decrease the efficiency of the knockdown. HIV-1 has a high mutation rate, and this is one of the reasons why RNA*i* gene silencing has not yet been shown to protect cells against HIV-1 in long-term virus replication assays although they were successful in the short term. For example, the effects of anti-HIV-1 siRNAs in lymphocytes were progressively dampened by the emergence of viral mutant genes *tat* and *nef* through nucleotide substitution or deletions within the siRNA target sequences.(44, 59) HIV-1 can also escape from RNA*i*-mediated inhibition through mutations that alter the local RNA secondary structure.(67) This emergence of escape mutants occurs even without necessarily changing the encoded protein after prolonged culturing. In order to circumvent the emergence of resistant viruses, targeting of conserved sequences and the simultaneous use of multiple siRNAs have been suggested. Further strategies to prevent this siRNA escape strategy by HIV-1 suggested the use of anti-sense for *tat* and *nef* genes. Unlike siRNA, the anti-sense approach is not a natural phenomenon occurring in the cells and no escape strategies have been developed by HIV-1. By the combination of gene knockout by two approaches, an effective and complete suppression of HIV-1 can be achieved.

8. Multi-targeted knockdown of HIV-1 genes

Although targeting a single HIV-1 sequence can result in strong inhibition of viral replication, it is likely followed by viral escape. Thus far, studies establishing the utility of siRNAs in suppressing HIV-1 infection failed in the long run because of the high mutation rate of HIV-1 replication. There are considerable challenges in achieving this long-term inhibition, preventing the transient success achieved from translating into clinical advantage. Therefore, approaches that not only target different stages of the viral life cycle but also simultaneously target specific sets of cellular genes that are needed for viral entry should be explored. In fact, it has been clearly demonstrated that the introduction of multiple siRNAs specific for HIV-1 could lead to viral RNA degradation and replication during different stages of the viral life cycle.(59) This multi-frontal RNA*i*-mediated attack on HIV-1 potentially inhibits the mutation escape mechanism. There have been several successful demonstrations of inhibition of HIV-1 replication using siRNA targeting distinct steps of the viral life cycle. HIV RNA in the post entry complex was successfully degraded abolishing the integration of proviral DNA when siRNAs targeted more than one region.(43) Dual-specific short hairpin siRNA constructs containing an intervening bridge, targeted against both receptors were demonstrated to successfully inhibit HIV-1 replication, thus demonstrating the practical utility of an siRNA multi-frontal attack on HIV-1.(60)

It has been previously established that if the length of siRNA exceeds 30 bp, there is an induction of nonspecific antiviral interferon responses.(33) Contrary to this belief, it was shown recently that this phenomenon might not be applicable to all sequences. Chang et al. generated 38 bp siRNAs that can induce targeted gene silencing of more than one gene without nonspecific antiviral responses. This structural flexibility of gene silencing with siRNAs needs to be further explored in order to achieve complete inhibition of HIV-1 by targeting simultaneously several regions.(68) By targeting two separate regions to knockout transcription of the gene *rev*, the highest degree of inhibition of viral replication was achieved.(69)

These newer drug designs had shown initial promise, but fell short of practical utility in providing adequate protection in every case. Since no effective therapy is currently available for prevention, new and innovative therapies are urgently needed to control, prevent and

eradicate HIV-1 disease. With this backdrop of HIV-1 drug development research, we propose to develop a cocktail of HIV-1 drug analogous to the current clinical use of combinations of antiviral drugs that target the reverse transcriptase and protease enzymes. These combinatorial approaches attacking multiple targets were designed essentially to prevent escape strategies observed by HIV-1 by various labs.
Analogous to the current clinical practice of HAART therapy, RNA*i* approaches should also be administered in a combined fashion to prevent HIV-1 escape strategies.

9. Limitations and hurdles of *in vivo* delivery of RNA*i*

Although RNA*i* mediated inhibition through siRNAs to knockdown HIV-1 genes in the laboratory has been successful, transfection of these purchased siRNA from commercial sources is impractical and has little value for translational work. siRNA is highly labile and often requires exceptionally high levels to achieve gene silencing *in vivo*. Further, the gene-silencing effect of siRNA is directly dependent on the number of molecules available in the cells, underscoring the need for development of plasmid vectors for the continuous synthesis of siRNA inside the cell. Current challenges facing the *in vivo* application of siRNA are the maintenance of duplex stability to avoid endonuclease degradation, need for improved delivery and the need to minimize immunological responses.(70) Though successful *in vivo* application of siRNA was demonstrated in liver through high-pressure tail vein injection in a murine model, its applicability to humans is limited.(71, 72) The quantity of siRNA necessary for efficient silencing is incompatible with scale-up to larger preclinical models.(73) Liposomal packaging(74) and chemical modification of the RNA and polyethylene glycol (PEG)(75) conjugated methods give stability to siRNA molecules, but still require large amounts of RNA*i* and are financially non-viable techniques. Hydrodynamic transfection of siRNA has been successful in targeting organs in mice, but this approach is not practical for clinical use. (76, 77)
One way to address this problem is to construct a siRNA sequence for insertion in a vector for intracellular expression of siRNA. Here the siRNA cassette is driven by RNA polymerase III promoter such as U6 that express sense and antisense strands separated by short "hairpin" RNAs (shRNAs) that are cleaved by the dicer to produce siRNA. In some cases both the sense and anti-sense siRNA strands are transcribed separately, which then hybridize *in vivo* to make the siRNA. Expression from a DNA plasmid or a viral vector such as shRNA enhances stability and safe delivery of siRNA apart from providing continuous production *in vivo*. It has been demonstrated that the transfection of human cells with plasmid encoding shRNA against HIV-1 *rev* drastically inhibited viral replication over a period of several days. Further, the highest degree of inhibition of viral replication was achieved by simultaneously targeting two distinct sites within *rev*.(51, 69)
Selection of highly accessible targets within the HIV-1 RNA genome should be determined first with antisense DNA oligonucleotide arrays before designing shRNA.(78, 79) There is recent evidence that the efficacy of siRNAs is similarly influenced by secondary structure in the target transcript.(80). These selection criteria should be used in the design of the siRNA molecule in order to get optimal inhibition.

10. Efficient delivery and expression of shRNA by viral vectors

One of the important limitations of siRNA-mediated drug delivery is the vehicle to carry the inhibitory molecules to the target cells. Viral vectors are generally more efficient vehicles for

shRNA *in vivo* than nonviral vectors.(81) Adenovirus, retro- or lentivirus, and AAV have been successfully used for this purpose. Retroviral/lentiviral vectors can potentially generate insertional mutagenesis, while adenoviral vectors trigger unacceptable levels of immune responses with concerns of safety.(82) shRNA can be packaged as recombinant viral vectors for better delivery in the whole organism. Retroviral vectors are successfully used for shRNA delivery, derived from moloney murine leukemia virus (MMLV). Lentiviral vectors are derived from HIV itself and can infect all the cells without the need for receptor interaction. Studies with lentiviral vectors silencing CCR5 have been performed but showed that the down regulating effect of CCR5 alone was insufficient. However, combinatorial constructs targeted to both CXCR4 and CCR5 and have shown better efficacy.(61) Retroviral/lentiviral vectors randomly integrate into the genome, generate insertional mutagenesis, and are derived from pathogenic viruses.

Dual-specific short hairpin siRNA constructs containing an intervening spacer, targeting receptor and co-receptor, demonstrated the practical utility of shRNA constructs synthesized as a single transcript. Since the shRNA design will permit tandem assembly of multiple motifs, it is now possible to introduce promising multivalent siRNA constructs into viral vectors for *in vivo* gene therapeutic applications. Based on this rationale, recent work with synthetic siRNAs demonstrated that down regulating either CXCR4 or CCR5 will protect cells from X4 or R5 HIV-1 strains, respectively, at the level of viral entry.(83) As mentioned earlier, CCR5 is a co-receptor, necessary for cellular entry by HIV-1 (R5 tropic viral strain), but is dispensable for normal human physiology. Owing to its crucial role in HIV-1 infection, the CCR5 co-receptor has been the subject of many therapeutic approaches, including RNA*i*-mediated gene silence therapy. siRNA targeting was shown to be effective; however, complete knockdown remained an elusive goal. In one study, transgenic macrophages expressing high levels of CCR5 were used for testing the efficacy of lentiviral vectors carrying CCR5 shRNA. Lentiviral delivery of longer (28-mer) shRNA were shown to be very effective in gene knockdown.(84) Thus, anti-CCR5 shRNA viral delivery is a promising candidate for clinical application.

We have tested retroviral vectors for gene therapy(85, 86); however, retroviral-mediated gene therapy is limited by a variety of practical and theoretical concerns, such as the immunogenicity of viral capsid proteins and insertional mutagenesis, which limit their utility for clinical purposes.(87)

Adenoviral vectors have also been successfully used for the delivery of shRNA(88) but they are well known to trigger unacceptable levels of immune responsiveness due to their large size and thereby limit repeat administration. Stability and efficiency is not the concern with viral vectors, but safety is a primary concern.

11. Recombinant Adeno-Associated Virus (rAAV) – Ideal RNA*i* gene silence therapy vectors

AAV belongs to the parvovirus family and is the only viral vector not known to be associated with any human disease(89) and the smallest vector suitable for RNA*i*-mediated gene silencing. Due to the safety(90), efficacy and potency provided by rAAV vectors, they make better alternative to the more commonly-used retroviral, lentiviral and adenovirus based vectors for gene therapy. rAAV vectors are easy to propagate and have many characteristics that make them a better choice for somatic gene therapy with RNA*i*-mediated gene silencing.(91, 92) rAAV vectors have long been established to transduce a wide variety

of tissues.(93-95) First generation rAAV vectors were single stranded, but the development of self-complimentary (double stranded) rAAV vectors helped to avoid delay in trans-gene expression.(96) Multiple administration of the rAAV vectors is possible to overcome neutralization by the antibody produced following the initial administration due to the availability of multiple serotypes with significantly higher trans-gene expression levels than that of prototype single stranded-vectors.(97) rAAV-based vectors have the potential for stable long-term trans-gene expression. rAAV is naturally gutless vector, which do not express any viral genes or cause a cytotoxic cellular immune response in the host. Furthermore, rAAV vectors show only a modest frequency of integration into host genome, thus avoiding insertional mutagenesis.(89) Overall, rAAV vectors fulfill the requirements for an ideal *in vivo* RNA*i* delivery vehicle.

12. Capsid mutant rAAV for enhanced transduction

One of the shortcomings of the traditional rAAV vectorology is low transduction efficiency, which requires large doses of vectors to achieve the desired effect. This is due to the phosphorylation of AAV capsids at tyrosine residues in the cell, which leads to a ubiquitin-proteasome-mediated destruction of the majority of rAAV particles and a decrease in transduction efficiency.(98) Gene therapy with these traditional rAAV vectors necessitates the delivery of undesirably high doses of the virus in order to achieve therapeutic benefit.(99) Recent advances have lead to the generation of rAAV vectors with mutant capsids protecting them from ubiquitin-proteasome-mediated degradation in the cytosol, eventually leading to an increase in DNA transduction efficiency.(7) We acquired these next generation rAAV vectors from Dr. Srivastava's laboratory consisting of a variant of rAAV-2/8 with a mutated capsid making the vector resistant to degradation in the cytosol.(7) By using triple-capsid mutant, pACG2-3M (Y444F, Y500F & Y730F)(7), along with the self-complimentary rAAV vector, we achieved a significant enhancement in rAAV2-sc green fluorescent protein (GFP) mediated transduction (Fig-1). With pseudo-typed rAAV vectors and capsid mutations, even greater *in vivo* transduction efficiency has been demonstrated.(7)

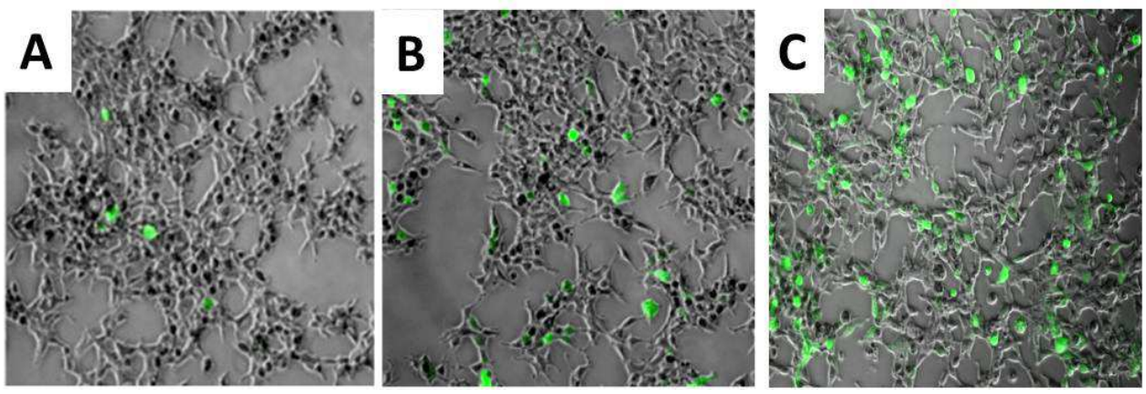


Fig. 1. HEK-293 cells transduced with various preparations of rAAV-GFP showing transduction efficiency based on green fluorescence intensity. A. Wild type rAAV-GFP; B. rAAV double stranded-GFP; C. rAAV-Capsid mutant.

13. rAAV vectors for siRNA delivery *in vivo* as short hairpin RNA (shRNA)

An ideal vector system for RNA*i* expression should be efficient and allow stable expression of the shRNA cassette without causing insertional mutagenesis or undesired immune responses. AAV is a small virus of 4.7 kb and relatively simple in its organization, comprising only two genes *Rep* and *Cap*. AAV vectors are extremely efficient tools for gene delivery *in vitro* and *in vivo*, as demonstrated by a number of laboratories. rAAV vectors have been shown to efficiently transduce hematopoietic cells.(100-102) Moreover, rAAV only retain inverted terminal repeats but do not express any viral genes and thus are gutless by design and definition more over it has not been associated with any human pathogenic, making it the vector of choice for human gene therapy. Because of their efficient transcription and inability to recombine with HIV-1, rAAV vectors represent a promising form of anti-retroviral gene therapy.(100)

One of the first studies using rAAV vector to deliver shRNA by Tomar et al. provided initial proof of principle that rAAV vector particles can be engineered to express shRNA.(88) They showed efficient knockdown of p53 and caspase 8 proteins. Subsequent studies by several investigators further demonstrated the usefulness of the rAAV vectors to express the shRNA cassette.(103-106) rAAV vectors have also been successfully used for a variety of gene silence experiments.(103-105) Use of rAAV vector encoding an anti-sense RNA against HIV-1 has been well documented by various labs.(23, 27, 100, 107) We studied the effect of anti-sense p53 gene transduction in a multiple myeloma cell line, ARH77, using AAV vector, where we delivered p53 cDNA in an anti-sense orientation.(108) *In vivo* studies in mice showed persistent knockdown of the target tyrosine hydroxylase in a Parkinson's disease model. They further demonstrated that reduction in the target elicited behavioral defects in the treated mice and created a phenotype reminiscent of rodent models of Parkinson's disease.

rAAV-mediated transduction is very efficient, particularly when compared with passive entry of simple siRNA or plasmid DNA.(109-111) rAAV siRNA delivery has been recently tested by several groups and shown to be highly efficient. Specific and efficient inhibition of HIV-1 replication was demonstrated in cultures.(91, 112) Together, this underscores the great promise of pseudo-typing shRNA-expressing AAV vectors to achieve targeted and controlled siRNA induction *in vivo*. Although the targeting of a single HIV-1 sequence can result in strong inhibition of viral replication, it is likely to be followed by viral escape. In fact, in most *in vitro* tests, siRNA did not stand the test of long-term protection against HIV-1. To overcome this escape strategy by HIV-1, we have a multi-pronged attack on HIV-1. First, HIV-1 is targeted at multiple genes for inhibition. Second, HIV-1 entry is inhibited by targeting siRNA to its cellular receptor CCR5 that is resistant to mutation. Third, we are introducing an anti-sense approach to knockdown HIV-1 *tat*, shown to be responsible for siRNA escape. Synthetic bi-specific or combinatorial constructs targeting both CXCR4 and CCR5 receptors have shown to confer resistance to HIV-1 infection much stronger than that conferred by targeting each one alone, giving a clear indication that multiple targeting is better than a single target.(22, 43, 61, 113)

14. Conclusion

Gene silencing therapy has the potential to inhibit HIV-1 replication and increase patient quality of life as an additional therapeutic class, and may serve as an adjuvant to current HAART treatment. This review gives a brief introduction regarding the emergence of RNAi,

the hurdles to overcome to proceed to the next stage, and possible solutions. Although RNA*i* molecules can be introduced into cells as double stranded or expressed from a plasmid to inhibit abnormally elevated genes, transfection of these purchased molecules from commercial sources is impractical and has little value for translational work. The main difficulty thus far in extending the power of RNA interference (RNA*i*) to clinical practice has been the development of safe vectors coding for shRNAs to achieve persistent knockdown *in vivo*. rAAV vectors are different from other vectors, since the only gene expressed from recombinant vector is the trans-gene itself, naturally gutless by design and thus avoiding any cytotoxic cellular immune responses in the host. Furthermore, rAAV vectors show only a modest frequency of integration into the host genome, thus avoiding insertional mutagenesis, which has been a stumbling block for the clinical use of retroviral or lentiviral vectors. Development of self-complimentary (also known as double stranded) vectors to avoid delay in trans-gene expression(96, 114) and packaging with capsid mutants(115) to increase transduction efficiency has further contributed to rAAV vectorology. Recent advances in our understanding of RNA*i* make rAAV an especially attractive candidate for anti-HIV-1 gene therapy, and rAAV-based RNA*i* approaches can be combined with other therapeutic modalities to make a combinatorial therapy akin to HAART.

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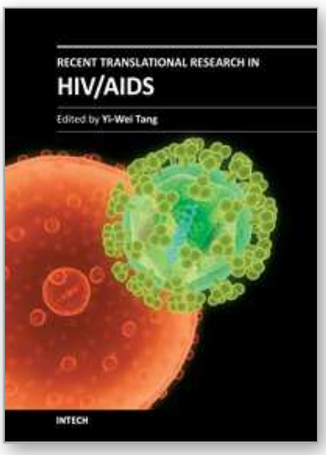
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The collective efforts of HIV/AIDS research scientists from over 16 countries in the world are included in the book. This 27-chapter Open Access book well covers HIV/AIDS translational researches on pathogenesis, diagnosis, treatment, prevention, and also those beyond conventional fields. These are by no means inclusive, but they do offer a good foundation for the development of clinical patient care. The translational model forms the basis for progressing HIV/AIDS clinical research. When linked to the care of the patients, translational researches should result in a direct benefit for HIV/AIDS patients.

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51000 Rijeka, Croatia
Phone: +385 (51) 770 447
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中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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