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Combinatorial RNA-based gene therapy for the treatment of HIV/AIDS

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Abstract

Introduction—HIV/AIDS continues to be a worldwide health problem and viral eradication has been an elusive goal. HIV+ patients are currently treated with combination antiretroviral therapy (cART) which is not curative. For many patients, cART is inaccessible, intolerable or unaffordable. Therefore a new class of therapeutics for HIV is required to overcome these limitations. Cell and gene therapy for HIV has been proposed as a way to provide a functional cure for HIV in the form of a virus/infection resistant immune system.

Areas Covered—In this review we describe the standard therapy for HIV/AIDS, its limitations, current areas of investigation and the potential of hematopoietic stem cells modified with anti-HIV RNA as a means to affect a functional cure for HIV.

Expert Opinion—Cell and gene therapy for HIV/AIDS is a promising alternative to antiviral drug therapy and may provide a functional cure. In order to show clinical benefit, multiple mechanisms of inhibition of HIV entry and lifecycle are likely to be required. Among the most promising anti-viral strategies is the use of transgenic RNA molecules that provide protection from HIV infection. When these molecules are delivered as gene modified hematopoietic stem and progenitor cells, long term repopulation of the patient's immune system with gene modified progeny has been observed.

Keywords

HIV; AIDS; Gene therapy; RNA interference; ribozyme; RNA decoy; CD34; hematopoietic stem and progenitor cells; lentiviral vector

1. Introduction

Acquired immunodeficiency syndrome (AIDS) is the disease caused by infection with the human immunodeficiency virus type 1 (HIV-1). According to the World Health Organization (WHO), there were 34 million people living with AIDS, with 2.7 million new cases and 1.8 million deaths worldwide in the year 2010 [1]. Most patients who present with viral infection experience a prolonged reduction in the level of CD4+ T-cells to <200 cells/

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Declaration of interests

None of the remaining authors have any competing interests to declare.

mm³ leading to a profound immunodeficiency. Patients most often suffer from opportunistic infection *pneumocystis jiroveci* or other AIDS defining illnesses such as Hodgkin's disease, non-Hodgkin's lymphoma, lymphocytic leukemia and other malignant and non-malignant sequelae. Contemporary therapeutic intervention is aimed at controlling viral replication and preserving immune function.

The current standard therapy for HIV-infected individual is daily administration of a cocktail of antiretroviral drugs that inhibit various stages of the viral life cycle, known as combination antiretroviral therapy (cART). Treatments include drugs of six classes: nucleoside/nucleotide reverse transcriptase inhibitors, protease inhibitors, non-nucleoside reverse transcriptase inhibitors, fusion inhibitors, integrase inhibitors and CCR5 antagonists. Current best practice includes starting a cocktail of at least 2 or more drugs from each of at least 3 classes of drugs and when patient CD4+ T-cell levels fall below 350/mm³. recent evidence indicates that initiation of drugs before patients become symptomatic may provide a greater benefit [2]. While effective in reducing morbidity, improving the quality of life and increasing median survival, cART is not curative. Patients with well controlled viremia on cART (<50 infectious units/mm³ of blood) are known to harbor a latent viral reservoir in resting CD4+ T-cells. When cART is interrupted, latently infected cells produce infectious virus which is rapidly followed by a loss of peripheral blood CD4+ T-cells and progression towards immunodeficiency. Additionally, the prolonged use of cART is associated with other clinical sequelae including neural, renal, hepatic and cardiovascular toxicity, diabetes, lipodystrophy and other metabolic abnormalities. Moreover, the widespread use of cART and a lack of patient compliance with drug treatment schedules have resulted in the development of viral variants (escape mutants) that are drug resistant. Together, these limitations of cART emphasize the need for a more comprehensive approach to HIV therapy. The current focus of the field is chemotherapeutic purging of the latent viral reservoir or "sterilizing" cure for HIV. However, production of an "HIV-resistant" immune system may be a more rapid road to a "functional" cure in which patients may harbor latent virus but are immunologically healthy and independent of cART. The latter is the topic of this review article with a special emphasis on RNA-based stem cell gene therapy.

2.1 Overview of the market

The use of small molecule inhibitors of HIV viral entry, integration and replication to treat HIV/AIDS has been extensively reviewed and will not be considered here. The reader is referred to several excellent reviews on the topic. Immunotherapeutic approaches to HIV eradication including vaccine and cell-based gene therapy are becoming important alternatives to cART and may result in a functional cure of HIV/AIDS by preventing the loss of CD4+ T-cells and (potentially) providing an effective immune clearance of viral reservoirs, recently reviewed in. Multiple vaccine trials have demonstrated safety and feasibility of delivering vaccine as well as the generation of strong humoral immune responses (antibodies) in healthy people who are at risk of contracting infections. One of the largest clinical trials of vaccine (RV144) ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT00223080) number, NCT00223080) was designed to prevent transmission of the virus from infected to "at risk" individuals and included more than 15,000 adult volunteers. The results demonstrated the safety of a combination of vaccine (prime and boost) and lowered the rate of HIV infection by approximately 31.2% compared with the control group (p=0.04). However, the vaccine was not capable of inducing a therapeutic benefit in previously infected volunteers [3]. Vaccine trials to date in HIV infected individuals have been unable to demonstrate objective improvements in disease outcome or reduction in morbidity and mortality. The primary reasons for failure include the inability to mount an immune response to protective viral epitopes and the rapid appearance of viral variants in response to the selective pressure of cART [4].

[5][6][7][8] Hematopoietic cell and gene therapy has been proposed as a treatment for HIV-infected individuals and is largely predicated upon the creation of a durable HIV-resistant immune system. The feasibility of this approach was elegantly demonstrated when an HIV+ patient with acute myeloid leukemia was transplanted with an HIV-resistant hematopoietic stem and progenitor cell (HSPC) graft as part of his leukemia therapy and was cured of his HIV. The stem cell graft was obtained from an HLA-matched, unrelated (allogeneic) donor who was also homozygous for a 32 base pair deletion in the chemokine receptor 5 (CCR5) gene. Cells with this deletion (CCR5 Δ 32/ Δ 32) do not express the HIV co-receptor and therefore cannot be infected with R5 tropic virus. Individuals who are heterozygous in the CCR5 Δ 32 allele show slower disease progression while homozygous individuals are resistant to HIV infection. After achieving complete hematopoietic reconstitution with the donor graft, the patient was able to suspend cART and remains aviremic (undetectable HIV using sensitive PCR methods) four years after treatment. While some argue that he may carry a sub-clinical or low-level latent viral load, he remains “virologically asymptomatic” in the absence of cART. Thus, remaining aviremic in the absence of cART is the current standard for what is considered a “sterilizing” cure for HIV/AIDS.

Although this type of stem cell transplantation therapy showed great success, it cannot be widely applied to the general HIV-infected population for several reasons. First, the frequency of homozygous CCR5 Δ 32/ Δ 32 genotype (~1% in Western European Caucasian population and absent in people with Asian and African descent) and appropriate HLA-matching is rare preventing the identification of appropriate donors for most individuals. Second, the myeloablative conditioning regimen used for allogeneic transplantation is a highly toxic procedure and therefore not indicated for non-malignant diseases. Finally, the risks of graft versus host disease in allogeneic transplantation are significant and also represent an unacceptable risk for non-malignant patients. Recent progress in hematopoietic cell and gene therapy for patients with both malignant and non-malignant indications has been reported (reviewed in) and has prompted several similar clinical trials for HIV therapy. As a more practical approach to allogeneic transplantation, multiple protein- and RNA-based antiviral strategies to confer resistance to HIV to T-cells or HSPCs have been developed by several groups (including our own) targeting viral genes and host factors required for viral replication (reviewed in). Knockdown or knockout of the required co-receptor CCR5 for viral entry by RNA interference, hammerhead ribozymes, and DNA-editing zinc finger nucleases (ZFNs) have been extensively studied. Alternatively, knockin strategies to introduce host restriction factors to block viral replication have also been reported. For example, the gp41-derived peptide (C46) blocks viral entry by preventing fusion. Other restriction factors, such as TRIM5 α from rhesus macaques (reviewed in), polynucleotide cytidine deaminase APOBEC 3G and 3F (reviewed in), and BST-2/tethrin (reviewed in), can also prevent HIV infection and expand the possible repertoire of molecular targets useful for gene therapy. Similarly, viral replication can be interfered in the transcription stage by targeting viral mRNA for degradation with RNA interference (e.g.,) and hammerhead ribozymes (e.g.,), or sequestering the transcription activator Tat protein by TAR RNA decoy, or alternatively blocking Rev-mediated transport by expressing dominant negative Rev mutant (RevM10), sequestering Rev protein by RBE RNA decoy, or degrading Rev mRNA by RNA interference. In this review, we describe our experience with the development of combinatorial approaches utilizing anti-HIV RNAs and the use of gene-modified adult hematopoietic stem cells in a pilot clinical trial in HIV patients undergoing autologous transplantation for lymphoma.

2.2 Introduction to Gene Transfer Strategies

Delivery of the therapeutic genes can be accomplished by non-viral transfection and viral-mediated transfer depending on the persistence of gene expression required. For example,

transient expression of ZFNs to create permanent gene disruption or knockout is efficiently achieved using non-integrating viral vectors (viral vectors that do not integrate into the host genome) and transient transfection. For most applications where knockdown of HIV or host factors are desired, persistent expression of the therapeutic genes is required. Lentiviral vectors are valuable gene delivery vehicles for this purpose based on their ability to transduce non-dividing cells (e.g., stem cells), integrate into the host genome and maintain long term expression of a transgene [9–12]. In our recent pilot clinical trial, an HIV-based DNA vector (pHIV7) [13], was utilized as a platform to express three anti-HIV small RNAs (Figure 1) [14][15][16]. pHIV7 has majority of the HIV genes removed to reduce the possibility of generating replication-competent helper virus to improve safety profile and therefore requires the co-transfection of multiple helper plasmids during packaging. Like other typical third-generation replication-defective and self-inactivating (SIN) lentiviral vectors, the 200-bp deletion in the U3 region in the 3' LTR is copied to the 5' LTR after reverse transcription resulting in transcriptional inactivation of both LTRs and generation of SIN vector (rHIV7) after integration into the target cells (Figure 1).

2.3 Expression, Mechanism of Action and Specificity of Inhibitory Small RNAs

Similar to the drug-based cART, anti-HIV genes should also be expressed in combination to reduce the frequency of viral escape. The clinical rHIV7-sh1-TAR-CCR5RZ lentiviral vector contains three anti-HIV small RNA with distinct mechanisms of action with synergy in inhibitory activity while prolonging antiviral protection (see Drug Summary Box) (Figure 1). The former two RNA agents are driven by the RNA polymerase III (Pol III) human U6 promoter while the last is driven by the Pol III VA1 promoter. The ubiquitous Pol III promoters ensure strong small RNA expression in all hematopoietic lineages.[17, 18] The sh1 small RNA gene encodes a short hairpin RNA (shRNA) that mimics the natural pre-miRNA substrate in the endogenous RNA interference pathway in generation of small interfering RNA (siRNA) targeting an overlapping region shared between Tat and Rev mRNA to mediate both transcripts for cleavage and protein knockdown. Tat and Rev are early regulatory HIV-1 proteins that are essential for viral replication [19][14, 20–23][8, 24–26][27] with Tat essential for efficient viral transcription and Rev essential for the export of intron-containing viral transcripts to cytoplasm for translation and packaging. Concerns related to potential toxicities due to saturation of the endogenous RNAi machinery and off-target effects have been minimized by careful design of the shRNA such that the intended siRNA strand is preferentially incorporated into RNA-induced silencing complex (RISC) to mediate knockdown and optimal balance between RNA expression and therapeutic effects. Microarray studies showed minimal perturbation of endogenous miRNA expression (unpublished data) while standard hematological functional assays demonstrated no siRNA toxicity as gene modified HPSCs had similar hematopoietic potential as unmanipulated cells [discussed further in Safety and Tolerability section below].

[14, 27][23]The TAR small RNA gene encodes a nucleolar-localizing RNA decoy aimed to trap the HIV-1 Tat protein in the nucleolus [22]. It contains the endogenous Box C/D U16 small nucleolar RNA (snoRNA) as a scaffold with the apical loop substituted with the minimal HIV-1 TAR structure. This chimeric RNA molecule co-localized with HIV-1 Tat in the nucleolus as demonstrated by *in situ* microscopy and exhibited potent antiviral activities. More importantly, nucleolar U16TAR decoy was more potent in inhibiting viral replication than its nuclear counterpart highlighting the importance and the potential of nucleolar trafficking of HIV for development novel antiviral therapeutics [22]. Because RNA decoys function in sequestering their intended targets, toxicity due to over-expression have not been reported.

Finally, the [5]CCR5RZ small RNA gene encodes a CCR5-targeting ribozyme that functions as a viral entry inhibitor. Ribozymes are self-catalytic RNA molecules that recognize their targets by standard Watson-Crick base pairing followed by mRNA cleavage resulting in knockdown of protein expression. The specificity of the ribozyme has been engineered by carefully selecting the target region unique to CCR5 and not to other members of the β -chemokine family, including the highly homologous CCR2. Cells that stably expressing The CCR5 ribozyme showed 70% reduction in CCR5 surface expression in the CCR5 over-expressing HOS-CD4-CCR5 cell line and delayed infection from R5-tropic HIV strain in monocytic PM1 cell line [28].

One of the potential concerns for CCR5-targeting therapies is the shift of selection to X4 tropic viruses (HIV-1 viruses that requires CXCR4 as co-receptor for entry). Although this is plausible, it motivates the inclusion of other inhibitory small RNAs against viral targets in the combinatorial approach to inhibit viral replication after infection. Hematopoietic CD34+ stem cell-derived monocytes carrying a single copy of this clinical lentiviral vector were protected against JR-FL strain of HIV-1 for 42 days in an *in vitro* viral challenge assay with released virions with less viral fitness.

2.4 Pharmacokinetics and metabolism

The gene-modified HSPCs rapidly engrafted and gave rise to multiple hematopoietic lineages *in vivo* (including T-cells and monocytes) with detectable (low) levels of shRNA expression in the bone marrow and peripheral blood for 24 months and more than 36 months respectively. An estimate of frequencies of gene modification (gene marking) was performed by qPCR analysis for vector-specific WPRE gene sequences normalized to the endogenous single-copy housekeeping gene (ApoB). DNA extracted from peripheral blood mononuclear cells (PBMCs) showed gene marking frequencies ranging between 0.02 to 0.32% (200 to 3,200 copies per 10^6 cells) in the 24 months follow-up period (medium length 18 months, ranges from 6 to 24 months) [29]. At 18 months, gene marking of T-cells, monocytes, B-cells, and granulocytes were detected in peripheral blood of one of the patients providing evidence for the transduction of stem and progenitor cells capable of sustaining long term multi-lineage hematopoiesis *in vivo* [29][29,30].

2.5 Safety and tolerability

The safety of genetic modification of HSPC was determined using *in vitro* clonogenic and bulk culture assays of hematopoietic potential [29]. transduced cells had similar frequencies and types of hematopoietic colony-forming units as the non-transduced (control) cells in standard clonogenic assays. Analysis of the progeny of gene modified HSPC derived from 4 week bulk culture (in growth and differentiation-promoting cytokines) showed no differences in the kinetics or magnitude of lineage development between transduced and non-transduced cells. Limiting dilution plating was used to isolate individual clones of cells derived from gene modified HSPCs to determine the number of integrated copies of transgene per cell. Six of the >500 growth-positive wells analyzed were found to contain WPRE sequences (consistent with bulk culture estimates of 1%). Out of these positive wells, transgene copies ranges from one to three copies per cell. These assays were used (in part) to satisfy regulatory requirements for demonstrating the safety and potency of the HSPC product.

The transplant procedure was well tolerated by the patients with minimal short-term toxicities consistent with standard autologous hematopoietic cell transplantation. Infused cells successfully engrafted by day 11 (absolute neutrophil count of >500 for 3 consecutive days) and had no product related severe adverse events. No evidence for clonal dominance or treatment related leukemia has been observed. The low level of gene marking observed *in*

vivo [29] was the result of a combination of low level transduction of HSPC plus dilution of the gene modified product with 50–100 fold more unmanipulated HSPC (safety requirement). Taken together, these data indicate that the treatment was safe and well tolerated and provide a basis for a larger efficacy study.

2.6 Regulatory affairs

The manufacture of cell therapy products is governed by US code of federal regulations (CFR) parts 210, 211, 600, 601, 610, 1271 and other applicable laws as enforced by the Food and Drug Administration. Cell products are considered “Biologics” (as opposed to Drugs) and are thus reviewed by the Center for Biologics Evaluation and Research (CBER). Autologous products must be harvested, processed and returned to each patient using a well controlled and traceable system. Systems designed to ensure product identity throughout the process are required to prevent product mixups or misidentification. All raw materials and manufacturing processes must meet with current Good Manufacturing Practices including retention of individual batch records and testing results. Ruminant animal materials are discouraged in the production of cell products and extensive testing of all cell lines and reagents used in manufacturing must include *in vitro* and *in vivo* assays for the presence of a wide range of viruses and infectious agents. Autologous products must also meet standard product requirements such as identity (defined phenotype), purity, safety (sterility, endotoxin free, mycoplasma free) and potency (viability, anti-viral activity, blood forming potential) to be qualified for clinical use. Stability testing, dose, route of administration, contraindications and patient eligibility criteria are all required components of the of each Investigational New Drug application filed with the FDA for these products. Prior to licensure, cell therapy products must be tested first for safety and efficacy and then in randomized clinical trials against a placebo or the standard of care in the field. Surrogate markers such as control of viremia and CD4+ T-cell recovery may suffice for obtaining licensure under accelerated approval for salvage therapy but approval of cell therapy products as a first line therapy will likely require demonstrable improvements in median survival.

3. Conclusions

Gene therapy approaches for the treatment of HIV/AIDS holds great promise for a functional cure of the disease. In our most recent study, patients with AIDS-related lymphoma undergoing autologous hematopoietic stem cell transplant were infused with gene modified, HIV resistant stem cells to evaluate the safety and feasibility of this approach. Although a low level of gene marking was observed, the feasibility of isolating, gene modifying and delivering a HSPC product was demonstrated. Additionally, this study demonstrated long-term expression of anti-HIV RNA sequences (>3 years) demonstrating the potential for a long lasting antiviral effect. Thus, the data suggest the transduction process for gene modification and the utilization of these modified cells in transplant for treatment AIDS-related lymphoma are both safe and feasible but the low of gene marking prevents clinical assessment of antiviral efficacy. It is estimated that at least 20% of gene modified HSPCs is required for antiviral inhibition based on large animal studies. Therefore strategies to increase gene marking frequency and/or selection of gene-modified cells are needed for efficacy of gene therapy. These strategies can include but are not limited to more efficient transduction process or transduction of more primitive hematopoietic stem cells capable of long term engraftment (e.g. CD34+/CD90+ [31, 32]), elimination of infusion of unmanipulated HSPC, *in vivo* enrichment of gene modified cells. Anecdotal observation of increased gene marking following cART interruption in this and other studies suggests that gene modified (HIV-resistant) cells are enriched under the “selective pressure [33]” of acute viremia. If this holds true, then cART interruption could become a component of the gene

transfer approaches to *in vivo* selection. An alternative method for selective pressure is the inclusion of P140K mutant of the O6-methylguanine DNA methyltransferase (MGMT^{P140K}) in the therapeutic gene construct. MGMT^{P140K} confers cellular resistance to alkylating agents such as BCNU or temazolamide. Large animal studies have demonstrated the potential to dramatically increase the percentage of gene modified cells in peripheral blood after transplant of MGMT^{P140K}-expressing hematopoietic stem cells and repeated administration of alkylating agent [34–37]. Finally, HSPC transplantation requires some form of cytoreduction to “make space” in the marrow for engraftment of the infused cells. The use of fully ablative conditioning regimens designed to eliminate residual tumor in this study are toxic and too risky for use in a non-malignant HIV population. Therefore, reduced intensity conditioning regimens must be developed for this therapy to be broadly applicable.

4. Expert Opinion

Combination antiretroviral therapy (cART) is an effective therapeutic intervention for HIV/AIDS but is not curative. Stem cell gene therapy is an attractive alternative to cART with the potential for a functional cure in the form of a HIV-resistant immune system. Substantial pre-clinical research and early stage clinical trials support the safety and feasibility of this approach. The use of lentiviral vectors encoding RNA-based antiviral genes in this context is promising and has been shown feasible in a pilot clinical study. However, significant improvements in the genetic modification of stem cells and stem cell transplantation procedures will be required to move this area of investigation into proof of concept studies.

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* of interest

** of considerable interest

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Drug summary box

Drug name	rHIV7-sh1-TAR-CCR5RZ
Phase	Pilot
Indication	AIDS-related lymphoma
Pharmacology description/ Mechanism of Action	Self-inactivating lentiviral vector expressing <i>tat/rev</i> small interfering RNA, nucleolar TAR decoy, and CCR5 ribozyme
Route of administration	Autologous hematopoietic stem cell transplantation
Pivotal trials	NIH Recombinant DNA (RAC) Human Gene Transfer Protocol #0508-725 NTC00569985

HIV-1 Genome

pHIV7-sh1-TAR-CCR5RZ DNA construct

co-packaging helper plasmids:

Integrated rHIV7-sh1-TAR-CCR5RZ lentiviral vector

Figure 1. Comparison of the HIV genome and pHIV7-based DNA construct and integrated self-inactivating lentiviral vector

We used pHIV7, an HIV-based DNA construct to package lentiviruses as a deliver vehicle for anti-HIV small RNAs. pHIV7 has a hybrid 5' LTR with the U3 region replaced with the cytomegalovirus (CMV) promoter and enhancer sequence to eliminate the need for HIV-1 Tat protein for transcription during packaging. pHIV7 has majority of the viral genes removed and therefore requires the co-transfection of multiple helper plasmids during packaging. The co-packaging plasmids encoding for HIV-1 Gag/Pol and Rev provide the structural genes required to produce viral particles, while Rev interacts with RRE to reduce intron splicing during packaging and facilitates the export of intron-containing transcripts.

The use of Vesicular Stomatitis virus glycoprotein (VSVG) as the envelop expands the tropism of the vectors in addition to allowing concentration via ultracentrifugation. The packaging signal (ψ) is important for encapsidation while the Rev-responsive element (RRE) is required for export of intron-containing transcripts out of the nucleus. The flap sequence is required for nuclear import of the vector cDNA after reverse transcription and is required for transducing non-dividing cells [15]. The woodchuck post-transcriptional regulatory element (WPRE) increases the expression of the transgene by promoting RNA export and/or polyadenylation [16]. Upon integration into the target cells, the 200-bp deletion in the U3 region in the 3' LTR, denoted $\Delta U3$, is further copied to the 5' LTR after reverse transcription generating a self-inactivating vector to increase biosafety. The small RNA genes are cloned in the multiple cloning site (MCS) of the vector with the Sh1 targeting *tat/rev* common exon driven by the U6 promoter, the nucleolar U16TAR decoy driven by the U6 promoter, and finally the CCR5 ribozyme driven by the VA1 promoter.