

FROM THE BENCH TO THE CLINIC: STORY AND LESSONS FROM VRX496, THE FIRST LENTIVECTOR EVER TESTED IN A PHASE I CLINICAL TRIAL

LAURENT HUMEAU

Department of Research and Development, VIRxSYS Corporation, 200 Perry Parkway, Gaithersburg, MD 20877, U.S.A.

E-Mail: laurent@virxsys.com

Received: 13th November 2006 / Published: 5th November 2007

ABSTRACT

Gene therapy for HIV-1 infection has been proposed as an alternative to antiretroviral drug regimens due to emerging drug resistance and toxicity that raises concerns about HAART as a long-term therapy. VIRxSYS has developed an HIV-based lentiviral vector platform for delivery of genetic therapies. For the first clinical application of the gene delivery technology, VIRxSYS created VRX496, a lentiviral vector expressing a 937-base long antisense against the HIV envelope gene. Along with the lentivector, a packaging vector, VIRPAC, was created based on a single plasmid approach for transient production. Many safety features were incorporated into VRX496 and VIRPAC. VRX496 pre-clinical efficacy was demonstrated *in vitro* by achieving high transduction efficiencies with stable gene transfer into human primary CD4⁺ T lymphocytes and by showing selective resistance to CD4 down regulation with over 4 logs (99.99%) of HIV replication inhibition in challenge assays using various X4, R5 or dual tropism strains of HIV. In December 2002, the US Food and Drug Administration approved the first ever Phase I clinical trial of lentiviral vectors in humans, testing the safety and tolerability of a single infusion of autologous HIV infected CD4⁺ T Cells transduced with VRX496. No adverse events due to the product were observed. Although the purpose of the Phase I clinical trial was to establish the safety of the

therapy, and the number of patients in the Phase I clinical trial is too small to make any conclusions with respect to efficacy, potential effects of VRX496 were observed in patients during the monitoring of their circulating CD4 counts and HIV viral load.

INTRODUCTION

HIV infects 38.6 million people worldwide [1]. It is predicted that in the absence of drastically expanded prevention and treatment efforts, at least 68 million people will die from AIDS between the years 2000 and 2020. In the United States, it is estimated that almost 1.2 million people are infected with HIV [1]. The mortality due to HIV/AIDS is estimated to be approximately 2.8 million deaths annually worldwide, and approximately 15,000 in the U.S. [1].

The current standard of treatment for HIV/AIDS is combination antiretroviral therapy (CART). This therapy typically consists of a triple “cocktail” of a nucleoside reverse transcriptase inhibitor (NRTI), a non-nucleoside reverse transcriptase inhibitor (NNRTI) and a protease inhibitor (PI). Although these cocktails have been successful in reducing viral loads, restoring immune function, and prolonging patients' lives they do not represent a cure, and there are concerns regarding adverse effects associated with long-term usage of CART. Specifically, a variety of metabolic disorders including HIV-associated lipodystrophy, central adiposity, dyslipidaemia, hyperlipidaemia, hyperglycaemia and insulin resistance have been reported as resulting from combination therapies [2–4]. These reactions, combined with complex and cumbersome dosing regimens, can have an adverse impact on patient adherence to therapy [5, 6]. Furthermore, poor adherence has led to an increased rate of HIV resistance, resulting in viral strains that have reduced sensitivity to the drugs [7, 8]. In fact, a subpopulation of HIV infected individuals has failed or is resistant to combination antiretroviral drug therapy. Currently 10–15% of newly-transmitted HIV is already drug resistant. Over the past decade, gene therapy for HIV-1 infection has been proposed as an alternative to antiretroviral drug regimens [9, 10].

Gene therapy technology has the potential to treat a variety of serious life threatening diseases, which currently have limited treatment options. The first retroviral vectors used for gene delivery were chosen for their ability to have large payloads cloned into them, their ability to integrate into chromosomes of target cells to establish long-term expression, and because they do not transfer virus-derived coding sequence [11]. Unfortunately, 3 of 10 patients treated for SCID-X1 with replication defective retroviral vector-mediated gene transfer developed leukaemia from insertional mutagenesis [12]. The construction of vectors engineered to be safer alternatives continues to be a focus of gene therapy research. Adenovirus and AdenoAssociated Virus (AAV) vectors have been used because, unlike retroviral vectors, they do not possess leukaemia causing factors and are able to infect non-dividing cells. This results in the expansion of the number of host cells they can infect. Unfortunately, Adenovirus and AAV vectors have short term expression from limited integration and can lead to immunogenicity and toxicity as seen in the death of a patient in a gene therapy trial at the University of Pennsylvania [13]. After several setbacks in

earlier gene therapy clinical trials, the field of gene therapy has made some advances in recent years. The emergence of a new class of vectors, lentiviral vectors have yielded some of the most promising vectors for clinical application to date. Lentiviral vectors were derived from the human (HIV), feline (FIV) bovine, (BIV) or equine (EAIV) immunodeficiency viruses. Lentiviral vectors are able to transduce dividing and non-dividing cells at a high efficiency [14]. They are able to integrate into the chromosome for long-term expression of therapeutic payload, while lacking the antigenicity of adenovirus and AAV vectors [15]. Safety concerns exist about the use of lentiviral vectors in humans since they are derived from immunodeficiency viruses. However, advances in pseudotyping, allowing lentiviruses to be packaged with different envelope proteins besides the immunodeficiency virus envelope, and vector construction, to reduce the possibility of RCL generation, have reduced these concerns [16, 17]. Also, recent studies have shown lentiviral vectors to be safer in terms of insertional mutagenesis since they have an insertion pattern similar to wild-type-HIV (wt-HIV), and leukaemia is not a recognized side effect of HIV infection even though cells have integrated virus for years [18, 19]. Lentiviral vectors have also been shown to express their payload more efficiently than other vectors, which often experience silencing of the payload genes in the host cells [20]. Thus, *in vitro* experiments demonstrated that β -thalassaemia could be successfully treated with lentiviral vectors, while retroviral vectors were unable to correct patients' bone marrow stem cells [21]. Similar evidence was also seen in CD34+ cells when lentiviral vectors were transduced into a rhesus macaque model, under conditions of minimal stimulation, ordinarily insufficient for transduction with murine retroviruses [22]. Thus, lentiviral vectors have been shown to be safer and more efficient delivery tools than retroviral vectors. Although a significant portion of research is focused on lentiviral vectors, and they appear to be the most promising vector for clinical application, (17% of the ASGT abstracts from 2006 focusing on gene therapy vectors were about lentiviral vectors), only five clinical trials with lentiviral vectors (three of which are sponsored by VIRxSYS) have been approved to date in the United States.

A number of different genetic vectors, retrovirus and lentivirus, and accompanying genetic antiviral payloads have been utilized to combat HIV-1, including antisense RNA, transdominant proteins, ribozymes, RNA decoys, and single chain antibodies [23, 24]. Antisense RNA targeted to wt-HIV RNA offers a significant advantage over several other genetic antiviral approaches since it is not a protein and thus not immunogenic. HIV-1 vectors expressing anti-HIV-1 genes have been previously described [25, 26]. One of the advantages of using HIV-1-based vectors over other vector types for HIV/AIDS gene therapy is they can transduce primary CD4 T Cells with very high stable efficiency without overt pre-stimulation with cytokines, as is needed with murine onco-retroviral vectors. Another advantage is the anti-HIV effect is a combination of the anti-HIV antisense payload and competition by cis-acting elements in the HIV-1 vector, (e.g., TAR and RRE decoys), with wt-HIV for factors of replication and encapsidation. Another advantage occurs by flanking the anti-HIV antisense with HIV sequences in the vector's backbone. Vector anti-HIV antisense RNAs are effectively targeted to the intracellular site of wt-HIV RNA accumulation, hence mediating their effective destruction. HIV-based vectors may provide several important advantages over current HIV combination therapies. HIV-1 vectors are likely to be less toxic than current combination drug therapies since the antisense genetic antiviral is

expressed only in those cells that become infected with wt-HIV. Finally, HIV vectors are predicted to be safe since no novel genetic sequences are introduced into the patient-subject, since no novel genes are contained in the HIV-1 vector. All the sequences present in the vector are derived from highly conserved regions of wt-HIV that would be almost certainly present in any given HIV-1-infected individual. The HIV sequences that were used to create VRX496 were derived in part from pNL4-3, a prototypic HIV-1 molecular clone that is derived from two North American strains of HIV-1.

VIRxSYS' goal is to improve anti-HIV therapy through the use of a novel HIV-1-based lentiviral vector. VIRxSYS produced a lentiviral vector to treat HIV/AIDS, VRX496 that contains a 937 nucleotide anti-HIV antisense sequence. The anti-HIV antisense sequence contained in VRX496 is targeted to the 5' end of the wt-HIV envelope (*env*) gene, which is necessary for virus replication. The anti-HIV antisense genetic antiviral payload is located upstream of a major splice acceptor site and is thus dependent upon the expression of Tat and Rev proteins that are provided by wt-HIV that infects vector transduced cells. Secondly, the length of the antisense is over 900 nucleotides long, making it difficult for wt-HIV to create resistant strains that are sufficiently fit to cause disease. Preclinical tests have demonstrated the promise of VRX496. To date, VIRxSYS has successfully created a lentiviral vector for the treatment of HIV/AIDS and is currently testing the therapy in multiple Phase II trials after successful completion of an earlier Phase I trial. Clinical trial results indicate primary human T Cells transduced with VRX496 interfere with wt-HIV replication lowering the HIV viral load in the subject, and improving the CD4+ T Cell count and immune response.

LENTIVIRAL VECTORS

Lentiviral vector construction

Since lentiviruses have the ability to transduce nondividing cells and integrate for long-term expression of payload, it has been a goal to make lentivirus vectors for efficient delivery of genetic material. The construction of a vector from infectious viruses has safety concerns, so the vectors have been engineered to incorporate safety features to avoid the generation of infectious material. The evolution of lentiviral vector construction has gone through several generations. The first generation of vectors were viral particles generated by expression of HIV-1 core proteins, enzymes, and accessory factors from heterologous transcriptional signals and the envelope of another virus from a separate plasmid [27]. The next generation had the HIV-derived packaging component reduced to *gag*, *pol*, *tat*, and *rev*, reducing the amount of sequences encoding critical virulence factors [28]. Further refinement led to a third generation that had the HIV-derived packaging component reduced to *gag*, *pol*, and *rev* with *rev* expressed in a separate expression plasmid [29]. Production of this virus only occurs in the presence of endogenous *rev* because the 5' LTR is replaced with a cytomegalovirus enhancer and *tat* expression is not necessary.

VRX496

VIRxSYS' gene therapy vector, VRX496, is an HIV-1-based lentiviral vector carrying a 937-nucleotide antisense sequence targeted to the HIV-1 envelope (*env*) gene (Fig. 1). It is a fully gutted vector and does not encode any complete viral proteins. VRX496 was meticulously designed with several safety features to limit the likelihood of a possible recombination event *in vivo* that could lead to a replication competent lentivirus, or RCL [30]. Codon degeneration was used to minimize homologous regions that could lead to recombination events between wt-HIV, VRX496, and the packaging construct VRX577 (VIRPAC). Furthermore, even in the unfortunate event that, a recombination should occur, a stop codon was introduced into the remaining VRX496 *gag* sequence while a pause signal and a cis acting ribozyme was engineered into VIRPAC to prevent read-through and packaging of the VSV-G RNA sequence into the lentiviral particles. The anti-HIV-1 antisense sequence is *tat* and *rev* dependent and is thus only expressed in transduced cells that are infected with wt-HIV. VRX496 is produced using a 2-plasmid system. The first plasmid construct encodes the VRX496 lentiviral vector genome. A second or "helper" plasmid DNA construct, VIRPAC, is introduced with the vector construct via calcium phosphate co-transfection of a certified 293 cell line to provide proteins *in trans* that are necessary for production of the VRX496 lentiviral vector particle.

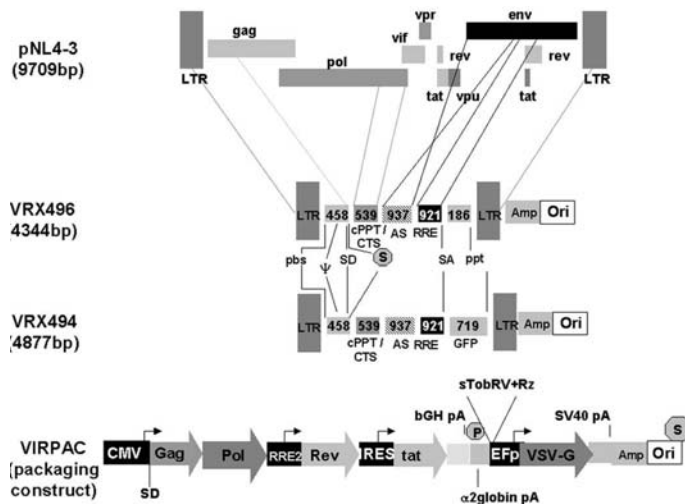


Figure 1. Schematic showing HIV_{NL4-3} and the fragments derived from it to make VRX496. Shown in stripes is the 937-base antisense sequence derived from a section of the envelope gene. Included in the vector are the HIV LTR for insertion and gene expression, and elements from *gag*, *pol*, and *env*, which encode the packaging signal (*psi*), the central polypurine tract (*cppt*), the *rev* response element (*RRE*) and a 186-base tag from GFP to allow differentiation between the vector and wt-HIV *in vivo*. A schematic of the packaging vector, VIRPAC is shown. A schematic of VRX494, a vector used in preclinical studies, is identical to VRX496 except for the fact that it expresses GFP instead of the GFP tag in VRX496, is also shown.

PRECLINICAL STUDIES

Gene transfer

To conduct preclinical studies of our vector, we created a VRX496 analogue expressing GFP, VRX494 (Fig. 1). In order to develop a therapy that would be effective, we first had to develop a protocol that would have efficient gene transfer into primary CD4+ T Cells. After fine-tuning our transduction protocol to maximize transduction efficiency in patient cells, we were able to reach transduction levels similar to those found in normal donor cells (>90%), far above what we were expecting (Fig. 2). All 17 patients tested using the optimized two-dose protocol were transduced at high efficiency. We were pleased to find that transduction efficiency was independent of patient viral load and CD4 count, indicating that our vector can modify CD4 T Cells from a range of patients. Although we could not test patients with a high viral load and high CD4 counts since they were unavailable for enrolment, we predict that these would respond similarly to those with high CD4 count and low viral load, based on the following logic: stimulation with iCD3/28 is critical to successful transduction efficiencies, and lower T cell responsiveness to iCD3/28 bead stimulation for expansion correlates with AIDS onset [31], which is defined by a CD4 T cell count of < 250 cells/mm³. Since a high CD4 T Cell count is not indicative of progression to AIDS, we would expect these cells to respond well to expansion and transduction. Since cells from patients with a broad range of CD4 counts and viral loads gave high transduction efficiencies using the optimized VRX496 protocol, this dual vector addition protocol has been implemented in our clinical trials.

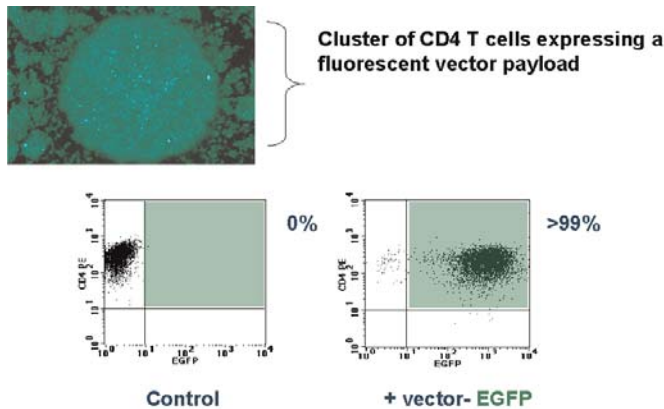


Figure 2. Flow cytometric analysis of CD4 and GFP expression in mock transduced CD4 T cells from HIV negative donors (control) or similar cells transduced at 20 TU/cell with VRX494 (+vector-EGFP), the GFP-expressing analogue of our clinical grade vector VRX496. Cells were seeded at 1×10^6 cells/ml in the presence of anti-CD3/28 antibody-coated beads, IL-2, and vector at an MOI of 20 TU/ml. GFP expression was measured at day 7-post-transduction.

HIV inhibition

The ultimate goal of high transduction efficiencies is to achieve a strong suppression of HIV in patient cells. Inhibiting productive HIV replication may prevent cell death of HIV-infected cells and will reduce spread of virus *in vivo*, both effective ways of delaying and/or preventing onset of AIDS. There are several ways to measure inhibition of HIV replication, the most common is quantitation of p24 protein production in culture supernatant. Additional methods include measurement of CD4 down-regulation [32], intracellular p24 staining, and selective expansion of transduced cultures over mock cultures. To get a robust and consistent characterization of the antiviral effect of VRX496 in a variety of patients, we examined each of these parameters separately. All methods consistently showed suppression of HIV replication in cells derived from all patients regardless of their viral load or CD4 count [33]. We observed a steady and continuous decrease in p24 production throughout the culture period in transduced patient cells compared to increasing p24 production in mock cells (Fig. 3). This correlated with reduced percentages of cells infected in transduced cultures, as detected by intracellular p24 staining, indicating that vector inhibition of virus replication also prevents spread of HIV in culture. Inhibition ranged from 93% – 100% between patients. Inhibition of p24 in culture supernatants directly correlated with lower percentages of cells infected with HIV in transduced cultures compared to mock cultures. The consistent and high level of inhibition of p24 secretion, a surrogate for HIV replication, observed in transduced patient cultures (on average 98% for each group, was independent of the patient's viral load or CD4 count. We found that VRX496 is capable of controlling replication of both CXCR4 (X4) and CCR5 (R5) strains of HIV among the patients tested in this study and therefore both clade B, X4 and R5, HIV strain-infected patients are good candidates for VRX496 T Cell therapy.

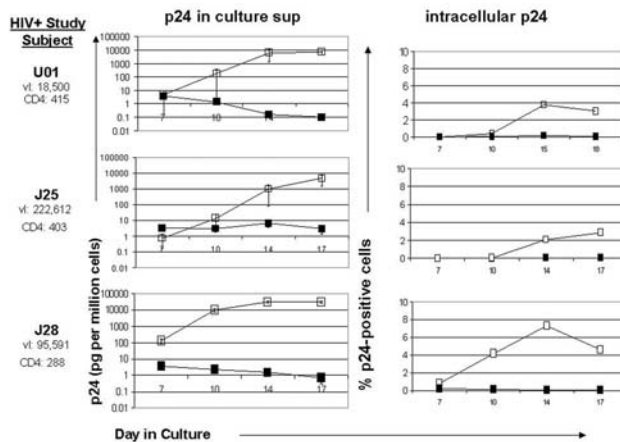


Figure 3. Inhibition of HIV replication as measured by cellular p24 secretion. Bi-weekly culture supernatants from mock (open symbols) and transduced (closed symbols) cultures from patient U01, J25, and J28 were taken from culture supernatant biweekly after bead removal from culture. Extracellular p24, a surrogate marker for HIV production, was measured by ELISA (ABL Inc) according to manufacturers

specifications. Measurement of intracellular p24, which quantitates the number of HIV-infected cells in a culture, was performed by flow cytometry after intracellular staining using the antibody KC57-RD1 from Caltag. Each mock and transduced culture was carried in triplicate, and each triplicate well was measured by ELISA in triplicates. Error bars represent SEM determined for each triplicate culture (where no bars are seen, the SEM was too small to be shown) using the average ELISA values for each triplicate. Since 10,000 events were collected in flow cytometry and the cells from the triplicate cultures were pooled, no error bars are shown for intracellular p24.

Selective advantage

To determine the selective advantage of VRX496 transduced cells we minimally transduced CD4+ T Cells isolated from healthy donors transduced with VRX494 to yield ~50% transduced cells in culture. We then infected the cultures at an m.o.i. of 0.05 with the R5 US1 wildtype strain of HIV. This culture mixture of transduced and nontransduced provides an environment where the untransduced cells are infected and the transduced/protected cells are constantly challenged. Transduced CD4+ T Cells exhibited a survival and selective growth advantage in culture after challenge with wt-HIV. We have established that increased copy numbers correlate directly with enhanced GFP mean fluorescent intensity (MFI) (unpublished data). At day 36, we observed a decrease in the number of nontransduced cells and a virtual disappearance of cells transduced at lower copy numbers, or MFI, with VRX494, while those transduced at greater copy numbers, or at a greater MFI, were enriched (Fig. 4). Therefore, the selective survival of cells *in vitro* with multiple copies capable of surviving an intense challenge illustrates the selective advantage conferred by the antisense-containing vector.

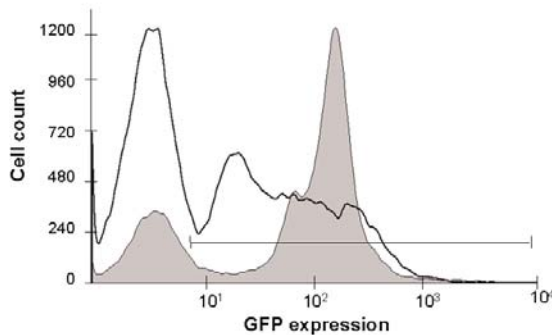


Figure 4. Selective advantage of vector transduced cells. Mean fluorescence intensity in partially transduced (50%) CD4-gated cultures 36 days following HIV infection (shaded line) or mock infection (open line).

HIV resistance

To determine if VRX496 creates mutant HIV strains resistance to VRX496 treatment we established an assay to generate viral breakthrough. Although viral breakthrough is not normally observed in vector-transduced primary human CD4 T lymphocytes, we were able to define conditions in the permissive SupT1 T Cell line to facilitate viral breakthrough. This was possible since SupT1 cells are more permissive to HIV replication than primary cells, in part due to their high level of division. Thus, these cells could replicate HIV to higher titres, improving the chance of development of potential escape mutant viruses. After infection of nontransduced SupT1 cells with HIV, peak p24 levels were observed 5 days later. However, in suboptimally transduced SupT1 cells, p24 levels did not peak until 20 to 25 days post-infection. Supernatants normalized for p24 and taken from normal and selected cultures were passaged serially three times for a total culture time of 56 days through transduced and nontransduced SupT1 cells. With each passage, selected virus was detected later than wt-HIV. After serial selection, p24 was produced earlier in selected virus than before selection, but at lower levels. This shift of the curve down and to the left suggests that a significant level of mutation may be occurring in response to antisense pressure.

To characterize the mutations that led to the change in HIV phenotype after vector-mediated suppression, a 2-kb region was amplified via PCR using wt-HIV-specific primers and then subcloned for sequencing. The mutations primarily fell into two categories: (i) those with large deletions in the envelope region, and (ii) those with only point mutations. The percentage of deletions in the breakthrough virus was much higher than in wt-HIV and the deletions always encompassed the antisense target region (Fig. 5A). The higher rate of deletion in virus from transduced cells indicated that antisense was exerting significant selective pressure on HIV to delete the region that binds the antisense region in the vector. Many of these deletions are quite large, which would result in severe debilitation of the virus. The ability of these mutants to persist with such defects likely resulted from co-packaging and/or pseudotyping with wt-HIV in cells. Alternatively, co-infection of complementing defective viruses in a cell could allow propagation of otherwise replication-incompetent viral species. Viruses that contained point mutations ranged from having background levels of mutations similar to that of wt-HIV to having high levels of mutations resulting in replication defective viruses. The mutation frequency was examined in the antisense-targeted region, the nontargeted region, and in the entire envelope region. The vast majority of the clones displayed similar mutation rates (ranging from 0.04 to 0.35%) to the wt-HIV control (ranging from 0 to 0.312%) (data not shown) over the entire region of the envelope. This suggests that the mutations in these clones may be equivalent to the spontaneous variations of wt-HIV. In addition, we conducted a *t* test on the mutation rate within the antisense target region compared to that outside the antisense target region and found that for those viruses exhibiting overall spontaneous mutation rates, there was no statistical difference between the regions ($P \geq 0.05$). Therefore, the selected viruses are predicted to behave similarly to wt-HIV, which is controlled by the antisense region expressed by our vector. However, three virus clones did have a significantly higher mutation rate in the envelope, BTP1, BTP2, and BTP3 (Fig. 5B). Significantly, these clones

only have increased rates of mutation within the antisense region and not outside the region complementary to the antisense, strongly suggesting that the mutations are a direct result of antisense-mediated selective pressure on the HIV genome. Due to the high percentage of base changes in these mutants, it was possible that the viruses would no longer be sensitive to antisense vector inhibition. To address this question, the functionality of the mutant envelopes was tested with respect to their ability to support viral replication and subsequent resistance to vector inhibition. To that end, the envelope regions from the mutant viruses were cloned back into the HIVNL4-3 backbone. The resultant viruses were tested for their replication ability by measuring the viral titres in terms of TCID₅₀ in nontransduced cells (Fig. 5B). Two of the three mutants were incapable of replicating in nontransduced SupT1 cells (BT-P1 and BT-P3). One mutant was able to replicate (BT-P2), but not to wt-HIV levels. These data indicate that the selected viruses are not increasingly virulent, but instead demonstrate a decrease in fitness or lost replicative ability. Finally, we examined whether these mutants had evolved to evade vector-mediated antisense inhibition. SupT1 cells were transduced with vector at 11 TU per cell, a suboptimal level that allows delayed replication of wt-HIV, and then subsequently challenged with wt-HIV or BT-P2 as a representative mutant capable of replicating. As a control, mock-transduced cells were infected with wt-HIV. As previously observed, in the absence of antisense, wt-HIV replicated to high titres between days 6 and 9 post-challenge. In transduced cells, wt-HIV p24 levels peaked at days 20 to 23, but BT-P2 was not able to replicate. These data support that the mutations acquired in the target envelope region of a selected virus capable of replication do not result in true escape mutants capable of evading the antisense therapy [34]. In summary, the length of the antisense is over 900 nucleotides long, making it difficult for wt-HIV to create resistant strains by deletion or multiple mutations of this region that result in a virus sufficiently fit to cause disease.

Abb. fehlt

Figure 5A. Table showing percentage of deletions in breakthrough virus compared to wt-HIV.

Abb. fehlt

Figure 5B. Mutant virus variants selected by antisense pressure are not fit. Viruses were produced by transfecting 293T cells with the mutant virus plasmids. Supernatants were collected at 48 hours post-transfection for determination of virus titre by TCID₅₀ in SupT1 cells. Actual values are as follows: 3.88×10^6 for wt HIV, 6.56×10^5 for BT-P2, and 0 for both BT-P3 and BT-P1 (ND is shown on the graph to indicate not detected). Mutation rates in the antisense region are shown in parenthesis. BT-P2 and BT-P3 were full-length envelopes that were inserted back into HIVNL4-3.

PHASE I CLINICAL TRIAL

The primary objective of our Phase I clinical trial was to determine the safety and tolerability of a single dose of autologous VRX496-modified T Cells in HIV positive subjects for 6 months post-dosing. A protocol amendment in March 2004 extended the study to include a 9 month post-dosing timepoint. To date, all 5 patients have completed the 9 month Phase I clinical trial and the 1 year follow-up safety visit.

Study synopsis

To be eligible for the study, subjects must have failed at least two HAART regimens as a result of drug resistance or be intolerant to antiretrovirals, with a viral load of ≥ 5000 copies per mL and CD4 T Cell counts between 150 and 500. In addition, subjects must have had a Karnofsky performance score of at least 80 and no signs of opportunistic infections. Potential subjects were excluded from participation in the study if they met any of the following criteria: pregnancy; history of cancer; history of class III or IV congestive heart failure; recent history of drug/alcohol abuse; receiving (within 30 days) corticosteroids, hydroxyurea, immunomodulating agents or antibiotics within 1 week of dosing. In addition, subjects with the following laboratory abnormalities were excluded: haemoglobin of less than 10 for males and less than 9.5 for females; absolute neutrophil count less than $1000/\mu\text{L}$; platelet count of less than $100,000/\text{mm}^3$; serum creatinine greater than 1.5 mg/dL; AST or ALT greater than 2.5 times the upper limit of normal; total serum bilirubin greater than 1.5 times the upper limit of normal; amylase and lipase outside normal range; and proteinuria of 2 or greater.

Subjects were monitored before dosing and at 3, 7, 14, and 21 days and at 3, 6 and 9 months post-dosing for viral load, CD4 count, emergence of potential replication competent lentivirus (RCL), and for immunological parameters. Adverse events were defined in part by observation of a sustained 0.5 log increase in viral load, or 30% decrease in CD4 count within 3 weeks post-dosing. Other safety measures were evaluated by physical examination (including HIV-specific physical examination) and by clinical chemistry, haematology and urinalysis.

Twenty-two subjects were screened between April 2003 and July 2004. Nine subjects failed to meet eligibility criteria and were not enrolled. Of the 13 eligible subjects, four withdrew consent and nine underwent aphereses. Four subjects did not proceed to dosing because of failure of T cell processing. The first patient was dosed in July 2003 and the fifth and last patient was dosed in September 2004.

Enrolled subjects

All subjects infused were male, aged between 27 and 45 years and had been diagnosed with HIV infection for a median of 15 years, with a range of 9-15 years (Table 1).

Table 1. Baseline subjects characteristics.

Characteristic	Subject				
	#1	#2	#3	#4	#5
Age	41	44	40	27	45
Gender	M	M	M	M	M
Ethnic Group	Caucasian	Caucasian	African American	African American	Caucasian
Baseline viral load	188,500	54,100	46,150	32,400	19,970
Baseline CD4 counts	253	316	273	308	220
HIV Infection (Yrs)	15	15	15	10	9
Discontinued Therapy ¹	4 NRTI + 2 NNRTI + 3 PI	5 NRTI + 4 PI	6 NRTI + 1PI	2 NRTI + 1 NNRTI + 1 PI	5 NRTI + 1 NNRTI + 1 PI
Therapy at Enrolment	2 NRTI + 2PI	3 NRTI + 1 NNRTI + 1 PI	None	2 NRTI + 1 NNRTI	2 NRTI + 1 PI

1 NRTI = nucleoside reverse transcriptase inhibitors; NNRTI = non-nucleoside reverse transcriptase inhibitors; PI = protease inhibitor.

2 Value determined at time of cell harvest.

3 RCL = replication competent lentivirus.

Mean viral load at screening ranged from 10,553 to 218,000 copies/mL, with a median viral load of 45,500. Mean CD4 counts ranged from 219.5 to 316 counts/ μ l with a median count of 273. At the time of screening all patients failed 2 or more HAART therapies (antiretroviral treatment history of 4 to 10 antiretrovirals). During the trial, the only subject not on any antiretroviral therapy was subject 3. None of the subjects changed their treatment during the first six months of the trial.

Subjects were infused after an independent DSMB reviewed safety data up to the 21-day post-dosing time point for the preceding subject plus cumulative safety data of all subjects. The first three subjects were dosed serially in this fashion. However, based on DSMB review and recommendations and IRB approval, subject 5 was dosed without a prior DSMB review for subject 4.

VRX496 T cell product

Ex vivo expansion of cell product was successful in five of nine subjects. Cells were expanded for a mean of 14 days yielding an average of 8×10^9 cells. The average copy number per cell for the five infused products was 2.08 (range 1.0 to 4.1). The purity of infused cells with respect to CD3 cells was 93.8%. All infused products tested negative for RCL (Table 2).

Table 2. T cell Product Description

Subject	#1	#2	#3	#4	#5
# VRX496 CD4 Cells Infused	1×10^{10}	1×10^{10}	0.6×10^{10}	1×10^{10}	0.9×10^{10}
%CD3 ⁺ Cells	95 ²	100	80	98	94
Avg. Vector Copy #	2.3	1.8	1.0	4.1	1.2
p24	< 50 ²	< 50	< 50	< 50	< 50
RCL ³	Negative	Negative	Negative	Negative	Negative

Safety

The single infusion was well tolerated and associated with minimal toxicity. No adverse events related to the product were reported during the infusion and vital signs were normal at the time of discharge. Six serious adverse events (SAEs) were recorded during the course of the trial but none were determined to be related to the product. All subjects underwent a scheduled apheresis procedure at the 6-month visit and a biological RCL test was conducted on the apheresed product. For the RCL test, patient cells were grown with C8166, which serves as an indicator cell line for 10 passages (five weeks) to allow sufficient amplification of a potential RCL. At the end of the amplification cycle, the culture was tested for the presence of VSVG DNA and VSVG RNA, by means of a TaqMan PCR and RT-PCR assay system. All tests were negative for VSVG DNA, and VSVG RNA signal or p24. Therefore, it can be concluded that no RCL was present in the subject's apheresed cells at the 6-month time point. Furthermore, VSVG DNA, VSVG RNA and VSVG antibody immunoassay were all negative at the 6 month post-infusion safety visit.

HIV-viral load and CD4 counts

A primary endpoint of the study was HIV viral load and CD4+ T Cell counts. We monitored the subject's viral load to ensure a sustained increase of greater than 0.5 logs was not achieved during the trial. We also monitored CD4+ T Cell counts to ensure that the patients CD4+ T Cells did not decrease by more than 30%. We did not encounter any safety problems during the trial. CD4+ T Cell counts remained relatively constant in three subjects (1, 2, and 5) with respective changes of -7, -34, and +5.5 cells/ml at 6 months compared to baseline. The other two subjects (3, and 4) saw much greater drops in absolute CD4+ T Cell counts at 6 months compared to baseline with decreases of -111, and -79 cells/ml, respectively. After month 6 we continued to see stable or increased CD4 counts in all subjects except 001 – 1022 (Fig. 6). The viral load was determined by the number of copies of HIV RNA in the blood, by Roche Amplicor assay. This assay had a variability of 0.5 log so stable decrease in viral load greater than 0.5 log was determined to be significant. A drop of 0.5 log from baseline values, was observed in three subjects; 1, 2, and 5, at Month 6. The viral load in the other two subjects (3, and 4) remained relatively unchanged from baseline at 6 months (0.02 log decrease and 0.3 log increase, respectively). After month 6 we observed decreases in the log change of viral load compared to baseline in all subjects except 001 – 1022 (Fig. 6). Although the purpose of the Phase I clinical trial was to

establish the safety of the therapy, and the number of subjects in the Phase I clinical trial is too small to make any conclusions with respect to efficacy, these results were very encouraging.

Abb. fehlt

Figure 6A. CD4 cell counts. CD4 cell counts are plotted as a change from baseline following the VRX496 infusion. Baseline values are shown in Table 1; baseline represents the average of two counts obtained during protocol screening. Subject #4 began antiretroviral therapy 7 months after infusion, and his viral load became undetectable at that point.

Abb. fehlt

Figure 6B. Log change in viral load from baseline. Viral load. The log plasma HIV viral load is depicted as change from baseline. The baseline values are shown in Table 1, and represent the average of two values obtained during the screening process. The VRX496 cell infusion was given on day zero. *Subject #4 began antiretroviral therapy 7 months after infusion, and his viral load became undetectable at that point.

Abb. fehlt

Figure 6C. Viral load data shown on a log scale for Patient 2 for up to 7 years prior to enrolling in the VIRxSYS Phase I trial. Regardless of changing drug regimens, this patient's viral load did not drop until after dosing with VRX496. The time of dosing is indicated by the arrow.

Pharmacokinetics and product metabolism in humans

There is no known information regarding the metabolism, absorption, plasma protein binding, distribution, or elimination of VRX496-modified T Cells in humans. However, persistence of the vector *in vivo* is being assessed by QRT-PCR with a sensitivity of 200 copies per million PBMC. Baseline samples were obtained approximately 15 minutes post-infusion. Persistence (up to the limit of detection) has ranged from 21 days for Subject 1 to 1 year for Subjects 2 and 4 (Fig. 7). At the 1-year time point, subject 4 has shown frequencies of engraftment of 0.04% (400 copies) after being undetectable at the 9-month time point. The half-life of VRX496 ranged from 11 to 46 days with a harmonic mean of 20 days. At day three, subject 001–1017 had a 3.5-fold increase in VRX496 persistence relative to baseline. A similar, albeit smaller increase was noted for subject 5 around this time period. The significance of this increase is unknown, but it could indicate migration of gene-modified cells from organ compartments [35, 36].

Abb. fehlt

Figure 7. Prolonged engraftment of lentiviral transduced CD4⁺ cells. Vector persistence was assessed beginning 20 minutes after infusion of VRX496 modified CD4⁺ cells. PBMC were collected at the indicated time points, and DNA analysis was performed for detection of VRX496 vector sequences using real-time PCR. The limit of quantification is 200 vector copies per 10⁶ PBMC. At the 1-year time point, Subject #4 has a frequency of engraftment of 0.04% (400 copies) after being undetectable at the 9-month time point.

Vector mobilization

Another potential safety concern associated with the use of lentiviral vectors is vectormobilization. Mobilization is possible for vectors that retain their full LTRs when packaging proteins are provided in trans such as during HIV infection. While vector mobilization *in vivo* to non-target tissues may have adverse safety consequences, mobilization of the vector payload into uninfected CD4⁺ cells could amplify the antiviral effects [25, 37]. Increased levels of GTAG RNA was observed in three subjects (2, 4, and 5) around 3 to 21 days following infusion (Fig. 8). Subject 4 had the highest increase. Coincidentally, he had the highest average vector copy per cell (4.0) in his infused product. Because of high RNase activity in plasma, RNA does not exist in the free state, unless it is protected within protein or lipid vesicles or associated with apoptotic bodies. Because GTAG RNA was not observed at baseline and was only evident after infusion of VRX496, a logical assumption is that GTAG RNA is associated with wt-HIV-1. A possible scenario is co-packaging with the wild type virus. From theoretical considerations the co-packaged unit is not expected to be replication competent. On the contrary, the replication deficient co-packaged unit is probably a prelude to mobilization of the vector. Mobilization to other CD4⁺ is the expected outcome with consequent propagation of anti-HIV effect by VRX496 vector.

Abb. fehlt

Figure 8. Vector Mobilization. VRX496 genomic RNA in the plasma was examined by assaying for the specific primer of green fluorescent protein (GTAG), which is a component of the vector. The G-Tag copies/ml plasma is graphed for each subject.

ELISPOT

To further elucidate the mechanism of action of VRX496 we evaluated the immune function of the patients by ELISPOT, which measures their immune response to HIV specific antigens (Fig. 9). The ELISPOT analysis shows a significant improvement in patient immune response in subjects 4, and 5 when exposed to HIV envelope antigens (9A), HIV gag antigens (9B), and diphtheria toxin (9C). These patients have a higher percentage of cells that secrete interferon gamma at three and six months post-infusion, then at baseline indicating a greater number of functioning T Cells are present.

Abb. fehlt

Abb. fehlt

Abb. fehlt

Figure 9. ELISPOT analysis of cellular memory responses in subjects treated with VRX496. Shown are responses to HIV envelope antigens (A), HIV gag antigens (B), and diphtheria toxin antigens (C). The bottom axis represents the patient number. Time-points evaluated are pre- dose (black), 3 months post-dose (white) and 6 months post-dose (red).

Integration studies

Integration site analysis was conducted on transduced T Cells harvested before infusion into the subject due to concerns raised during previous trials about insertional mutagenesis [12]. VRX496 was shown to integrate into similar types of genes at a similar frequency to wild type HIV (Fig. 10). Since the integration pattern of VRX496 was shown to be similar to wt-HIV it is believed this indicates a reduced risk of insertional oncogenesis compared to previously used retroviral vectors since leukaemia is not a side effect of HIV even though T cells may contain integrated virus for years [19].

Abb. fehlt

Figure 10. Integration profile of VRX496. The integration profile of VRX496 is compared to the integration profile of wt-HIV and a profile for random integration events.

Phase I trial conclusions

The Phase I safety and tolerability trial in five HIV positive subjects failing at least two HAART regimens provides data indicating that VRX496 based lentiviral vectors are safe. An independent Data Safety Monitoring Board has monitored this trial on a patient-by-patient basis, and all safety objectives have been met and no stopping rules encountered. There were no unexpected toxicities and no serious adverse events related to the product. Furthermore, there are indications of antiviral effects, even though the dose of VRX496 transduced T Cells was relatively low. Based on the safety profile demonstrated in Phase I, we have initiated a Phase II trial to evaluate the safety and tolerability of multiple infusions of VRX496.

SUMMARY

VIRxSYS has constructed a novel HIV-based lentiviral vector, VRX496, with built in safety features. The first clinical application of VRX496 was to treat HIV/AIDS. No RCLs have been generated and no insertional mutagenesis has developed, which are the major concerns with any gene therapy treatment. VRX496 has persisted for almost two years in the patients and the patients are experiencing significant decreases in viral load, stable or increased CD4 counts, and improved immune responses as measured by ELISPOT. The preclinical studies and Phase I clinical trial conducted to date to evaluate VRX496 has demonstrated the safety and feasibility of VRX496 as an HIV/AIDS treatment. Continued evaluation of VRX496 will not only determine the efficacy of the treatment, but will help identify the utility of gene therapy as an alternative treatment for HIV and other serious diseases. VRX496 is currently being evaluated in a multiple dose Phase II clinical trial in patients failing antiretroviral therapy. Trials are being planned to evaluate VRX496 in well-controlled patients and treatment naïve patients.

ACKNOWLEDGEMENTS

As the invited speaker that presented this material, I would like to acknowledge that without the contributions of others this work would not have been possible. I would like to thank our collaborators at the University of Pennsylvania: Bruce Levin, Frederick Bushman, Jean Boyer, and Carl June. I would like to thank the principal investigator of the Phase I clinical trial Rob Roy MacGregor. I would also like to acknowledge other contributors from VIRxSYS Corporation: Tessio Rebello, Arvind Chopra, Cathy Afafe, Xiaobin Lu, Boro Dropulic and Vladimir Slepushkin.

REFERENCES

- [1] UNAIDS (2004) *Report of the Global HIV/AIDS Epidemic*. pp. 1–95. UNAIDS, Geneva.
 - [2] Vigouroux, C. *et al.* (1999) Adverse metabolic disorders during highly active anti-retroviral treatment (HAART) of HIV disease. *Diabetes Metab.* **25**:385–392.
 - [3] Behrens, G.M., Stoll, M., Schmidt, R.E. (2000) Lipodystrophy syndrome in HIV infection: what is it, what causes it and how can it be managed? *Drug Saf.* **23**(1):57–76.
 - [4] Powderly, W.G. (2002) Long-term exposure to lifelong therapies. *J. Acquir. Immune Defic. Syndr.* **29** Suppl 1: S28–40.
 - [5] Lucas, G.M., Chaisson, R.E., Moore, R.D. (1999) Highly active antiretroviral therapy in a large urban clinic: risk factors for virologic failure and adverse drug reactions. *Ann. Intern. Med.* **131**(2):81–87.
 - [6] Max, B., R. Sherer, R. (2000) Management of the adverse effects of antiretroviral therapy and medication adherence. *Clin. Infect. Dis.* **30** Suppl 2: S96–116.
 - [7] Nijhuis, M., Deeks, S., Boucher, C. (2001) Implications of antiretroviral resistance on viral fitness. *Curr. Opin. Infect. Dis.* **14**(1):23–28.
 - [8] Turner, B.J. (2002) Adherence to antiretroviral therapy by human immunodeficiency virus-infected patients. *J. Infect. Dis.* **185** Suppl 2: S143–151.
 - [9] Sarver, N., Rossi, J. (1993) Gene therapy: a bold direction for HIV-1 treatment. *AIDS Res. Human Retroviruses* **9**(5):483–487.
 - [10] Dropulic, B., Jeang, K.T. (1994) Gene therapy for human immunodeficiency virus infection: genetic antiviral strategies and targets for intervention. *Human Gene Ther.* **5**(8):927–939.
 - [11] Trono, D. (2000) Lentiviral vectors: turning a deadly foe into a therapeutic agent. *Gene Ther.* **7**(1):20–23.
 - [12] Hacein-Bey-Abina, S. *et al.* (2003) LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* **302**(5644):415–419.
 - [13] Marshall, E. (1999) Gene therapy death prompts review of adenovirus vector. *Science* **286**(5448):2244–2245.
 - [14] Wiznerowicz, M., Trono, D. (2005) Harnessing HIV for therapy, basic research and biotechnology. *Trends Biotechnol.* **23**(1):42–47.
 - [15] Thomas, C.E., Ehrhardt, A., Kay, M.A. (2003) Progress and problems with the use of viral vectors for gene therapy. *Nature Rev. Genet.* **4**(5):346–358.
-

-
- [16] Wilson, J.M. (2004) Adeno-associated virus and lentivirus pseudotypes for lung-directed gene therapy. *Proc. Am. Thorac. Soc.* **1**(4):309–314.
- [17] Kaikkonen, M.U. *et al.* (2006) Truncated vesicular stomatitis virus G protein improves baculovirus transduction efficiency *in vitro* and *in vivo*. *Gene Ther.* **13**(4):304–312.
- [18] Montini, E. *et al.* (2006) Hematopoietic stem cell gene transfer in a tumor-prone mouse model uncovers low genotoxicity of lentiviral vector integration. *Nature Biotechnol.* **24**(6):687–696.
- [19] De Palma, M. *et al.* (2005) Promoter trapping reveals significant differences in integration site selection between MLV and HIV vectors in primary hematopoietic cells. *Blood* **105**(6):2307–2315.
- [20] Ellis, J. (2005) Silencing and variegation of gammaretrovirus and lentivirus vectors. *Human Gene Ther.* **16**(11):1241–1246.
- [21] Puthenveetil, G. *et al.* (2004) Successful correction of the human beta-thalassemia major phenotype using a lentiviral vector. *Blood* **104**:3445–3453.
- [22] An, D.S. *et al.* (2001) Lentivirus vector-mediated hematopoietic stem cell gene transfer of common gamma-chain cytokine receptor in rhesus macaques. *J. Virol.* **75**(8):3547–3555.
- [23] Dropulic, B., Jeang, K.T. (1994) Intracellular susceptibility to ribozymes in a tethered substrate-ribozyme provirus model is not predicted by secondary structures of human immunodeficiency virus type 1 RNAs *in vitro*. *Antisense Res. Devel.* **4**(3):217–221.
- [24] Veres, G. *et al.* (1998) Comparative analyses of intracellularly expressed antisense RNAs as inhibitors of human immunodeficiency virus type 1 replication. *J. Virol.* **72**(3):1894–1901.
- [25] Dropulic, B., Hermankova, M., Pitha, P.M. (1996) A conditionally replicating HIV-1 vector interferes with wild-type HIV-1 replication and spread. *Proc. Natl Acad. Sci U S A* **93**(20):11103–11108.
- [26] Dropulic, B. (1998) Novel lentiviral vectors for human gene therapy. *Expert Opin. Investig. Drugs* **7**(6):997–1001.
- [27] Naldini, L. *et al.* (1996) *In vivo* gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* **272**(5259):263–267.
- [28] Zufferey, R. *et al.* (1997) Multiply attenuated lentiviral vector achieves efficient gene delivery *in vivo*. *Nature Biotechnol.* **15**(9):871–875.
- [29] Dull, T. *et al.* (1998) A third-generation lentivirus vector with a conditional packaging system. *J. Virol.* **72**(11):8463–8471.
-

- [30] Lu, X. *et al.* (2004) Safe two-plasmid production for the first clinical lentivirus vector that achieves >99% transduction in primary cells using a one-step protocol. *J. Gene Med.* **6**(9):. 963–973.
 - [31] Roos, M.T. *et al.* (1998) Low T-cell responses to CD3 plus CD28 monoclonal antibodies are predictive of development of AIDS. *Aids* **12**(14):1745–1751.
 - [32] Benson, R.E. *et al.* (1993) Downregulation of cell-surface CD4 expression by simian immunodeficiency virus Nef prevents viral super infection. *J. Exp. Med.* **177**(6):1561–1566.
 - [33] Humeau, L.M. *et al.* (2004) Efficient lentiviral vector-mediated control of HIV-1 replication in CD4 lymphocytes from diverse HIV+ infected patients grouped according to CD4 count and viral load. *Mol. Ther.* **9**(6):902–913.
 - [34] Lu, X. *et al.* (2004) Antisense-mediated inhibition of human immunodeficiency virus (HIV) replication by use of an HIV type 1-based vector results in severely attenuated mutants incapable of developing resistance. *J. Virol.* **78**(13):7079–7088.
 - [35] Ho, M. *et al.* (1993) A phase 1 study of adoptive transfer of autologous CD8+ T lymphocytes in patients with acquired immunodeficiency syndrome (AIDS)-related complex or AIDS. *Blood* **81**(8):2093–2101.
 - [36] Brodie, S.J. *et al.* (1999) *In vivo* migration and function of transferred HIV-1-specific cytotoxic T cells. *Nature Med.* **5**(1):34–41.
 - [37] Weinberger, L.S., Schaffer, D.V., Arkin, A.P. (2003) Theoretical design of a gene therapy to prevent AIDS but not human immunodeficiency virus type 1 infection. *J. Virol.* **77**(18):10028–10036.
-