Selection and Characterization of Human Immunodeficiency Virus Type 1 Variants Resistant to Compounds that Inhibit Rev-RRE Function

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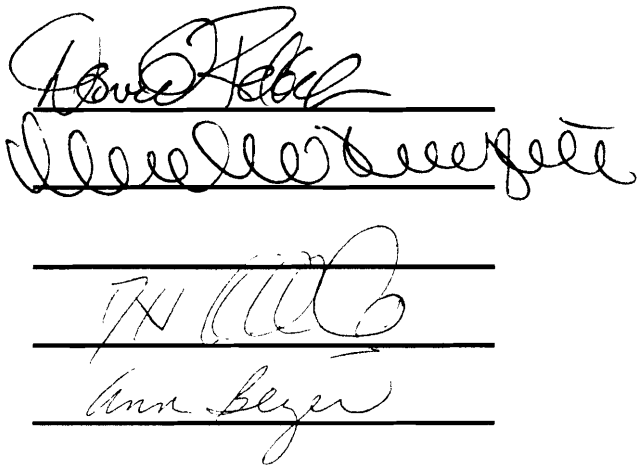

Abstract

The Human Immunodeficiency Virus Type-1 (HIV-1) uses the Rev-RRE pathway to export its genome and mRNAs containing introns. The interaction between the Rev protein and the Rev Responsive Element (RRE) presents a target for anti-HIV therapeutics. We studied the Rev-RRE pathway by utilizing small heterocyclic compounds shown in previous research to inhibit Rev function. By identifying and characterizing mutations in resistant NL4-3 variants, we were able to propose and test resistance mechanisms. Analysis of the resistant variants revealed mutations in the RRE. These mutations caused structural changes in RRE stem loop IIC, and also changed the amino acid sequence of gp41.

To separate the possibilities contributing to resistance, we utilized site mutagenesis to create point mutations that conserved the amino acid sequence conferred by the resistance mutations, but disrupted the structure of the RRE. The disrupted RREs caused the viruses to become sensitive to the compounds. Therefore the cause of the resistance was the structural changes in the RRE, not the changes in gp41. We also tested the wild type and mutant RREs in transient transfections both in provirus and pCMVGagPol constructs. These experiments indicated that the mutant RREs function at lower Rev levels than the wild type RRE.

We then performed selection studies using a HXB2 derivative virus, R73, with a differing genetic background from NL4-3. Instead of RRE mutations, we recovered changes in gp120 Env that conferred resistance.

Further selection studies with NL4-3 using both enfuvirtide (T-20), and 103833 or 104366, created variants with changes in RRE stem loop IIC and stem loop V. The change in stem loop IIC, one of our previously identified Rev-RRE compound resistance mutations, did not confer T-20 resistance. A silent mutation in stem loop V caused T-20 resistance. We hypothesized this altered the expression of the envelope message, overcoming the competitive inhibitor T-20 at the tested concentration.

Together the data suggest that different HIV-1 viruses function with optimized Rev levels for interaction with their RRE; and that they are able to adapt Rev-RRE interaction to maintain viral replication. Also additional mutations in *vpu* or *env* can contribute to resistance through separate mechanisms.

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Chapter 1

Introduction

HIV-1

Human Immunodeficiency Virus Type-1 (HIV-1) belongs to the genus of retroviruses known as Lentiviruses. HIV-1 is also recognized as the causative agent of Acquired Immunodeficiency Syndrome (AIDS) in humans. It is believed that in the early 1900's this virus evolved from a Simian Immunodeficiency Virus (SIV) that infects chimpanzees in central Africa (139, 144). With socioeconomic changes that occurred after World War II, HIV-1 gained access to expanding human populations causing an epidemic in Africa in the 1950's. The epidemic has spread throughout the world and currently afflicts over 40 million people, with the majority concentrated in the area of Sub-Saharan Africa (15, 71).

HIV-1 is the predominant primate Lentivirus affecting humans. HIV-2, a Lentivirus found also in gorillas, infects humans causing a less severe disease course. The virion structure, genetic composition, and replication cycle define Lentiviruses (92). Morphologically they have a cylindrical or cone-shaped core. They have complex mode of gene expression, translating fully spliced mRNA encoding regulatory proteins (Tat, Rev, and Nef), and partially spliced and unspliced mRNA encoding proteins that constitute the viral particle or provide accessory functions (11, 75).

HIV-1 is separated into distinct genetic subtypes or clades based on differences primarily in the *env* gene that encodes for the viral

envelope proteins, and the *gag* gene that encodes the viral matrix, capsid, and nucleocapsid proteins (32). HIV-1 isolates from different clades can differ by 40% in the amino acid sequence of gp120 Env. The genetic diversity of the virus is due to its ability to adapt to selective pressures from the host (155).

Viral Life Cycle

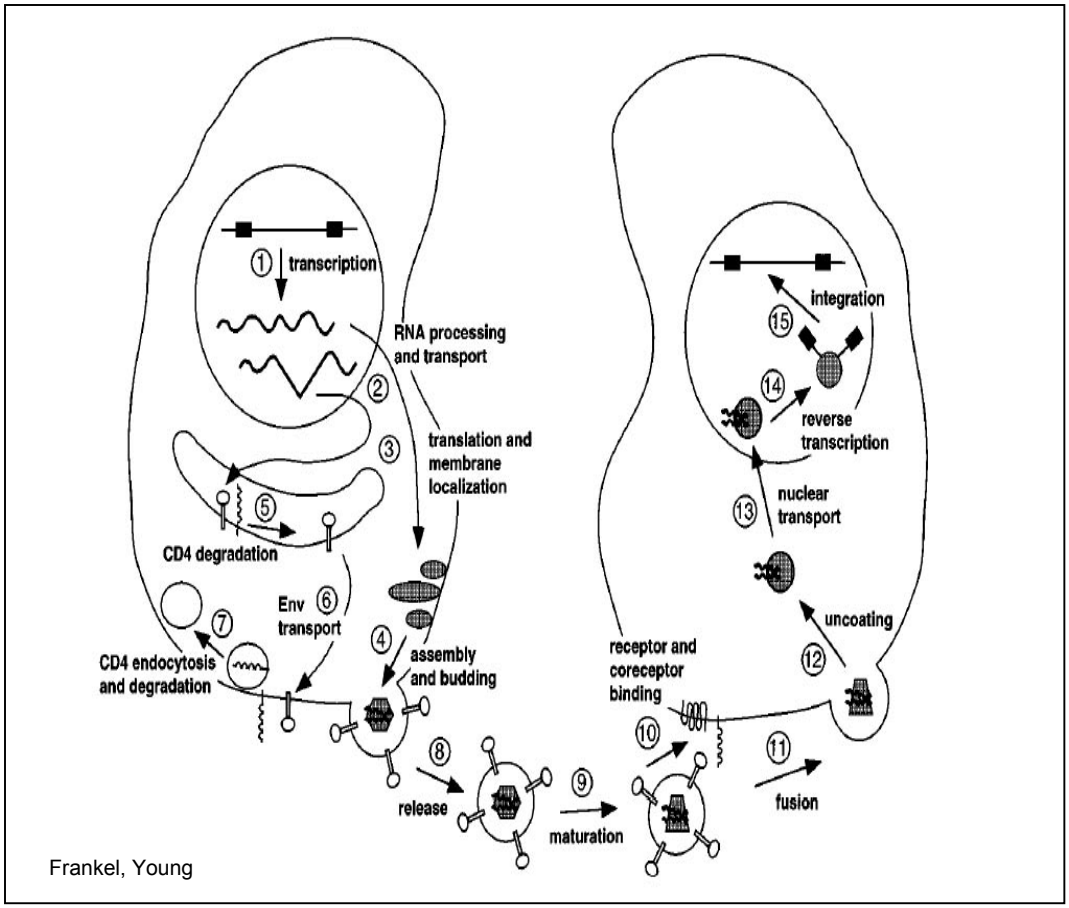
HIV-1, after entry into the human body through the blood or reproductive fluids, binds to host cell receptor molecules on target cells using viral envelope proteins. After fusion with the host cell membrane, the viral core is deposited into the cytoplasm. The proteins of the viral core disassemble and the viral RNA genome undergoes reverse transcription by the viral enzyme Reverse Transcriptase to produce double stranded DNA. This DNA enters the host cell nucleus within a viral preintegration complex, and then undergoes integration into the host cell genome. Integration is catalyzed by the viral enzyme Integrase, and host cell factors (4). The HIV-1 DNA that is integrated into the host DNA is called the provirus (61).

Transcription of the proviral DNA by the host cell RNA Polymerase II complex produces a full length RNA transcript that becomes alternatively spliced. The alternatively spliced transcripts are transported from the nucleus by two different transport mechanisms. Fully spliced messages transport by normal cellular mechanisms, and are translated to

form the Tat, Rev, and Nef regulatory proteins. The viral genome and unspliced and incompletely spliced messages utilize the viral Rev-RRE pathway (68). After transport, full length RNA is packaged as the new viral progeny genomes, or translated by cytoplasmic ribosomes to form the Gag and Gag-Pol polyproteins. These polyproteins are eventually cleaved by viral Protease into components of the viral particle and the viral enzymes (66).

Several different singly spliced species of mRNA are translated to form the accessory proteins Vif, Vpr, Vpu, as well as the structural protein Env. Env is translated by ribosomes bound to the endoplasmic reticulum (ER). This gp160 precursor protein is then transported through the Golgi apparatus, where it is glycosylated and cleaved to form the mature gp120-gp41 complex (101). Trimeric Env proteins are delivered to the surface of the infected cell where the viral RNA genome, Gag-Pol polyprotein assemble at budding sites on the inner face of the plasma membrane (72). Viral progeny bud from the infected cell and then undergo maturation. Maturation requires the action of the viral enzyme Protease, which cleaves the Gag-Pol polyprotein, causing the condensation of the viral core into a mature infectious virion (70).

Figure 1 illustrates the HIV viral life cycle from the time point of replication of the provirus already integrated into the cell to the infection and integration into another target cell.



Frankel, Young

Figure 1. HIV Viral Life Cycle.

The integrated provirus is transcribed into mRNA (unspliced, incompletely spliced, and fully spliced) (1) that is transported to the cytoplasm by separate mechanisms (2). The mRNAs are translated into the proteins of the viral progeny. Structural proteins of the core assemble along the inner face of the cytoplasmic membrane of the host cell (3). Proteins that comprise the Env heterotrimer are transcribed from ribosomes associated with the endoplasmic reticulum. These proteins are assembled and transported through the Golgi for placement in the host cell membrane (6). Viral progeny bud from the host cell (4) and are released into the extracellular fluids (8). The virus undergoes a maturation process by condensation of the viral core (9). This now infectious virus binds to the host cell receptor and coreceptor molecules (10) triggering fusion with the host cell membrane (11). The viral core is released into the cytoplasm (12), uncoating as it is transported to the host cell nucleus (13). The viral RNA genome undergoes reverse transcription (14) to produce DNA that is integrated into the host cell genome (15). At this point HIV exists as a provirus.

Introduction to HIV-1 Therapeutics

Vaccine development for HIV has proven difficult. A prophylactic vaccine that could prevent infection of an individual exposed to HIV would be ideal. However HIV has a high mutation rate, coupled with a rapid rate of replication, which allows the virus to produce variants capable of overcoming the various forces that are trying to eliminate it in the body (32, 65). A vaccine that could neutralize HIV has yet to be designed (31, 101, 106, 107). In addition to being highly mutable, the virus is also able to manipulate both the host cell it infects, and the host immune response, in order to escape neutralizing immunity (18, 65).

A therapeutic vaccine that only reduces the viral load in a patient with HIV is another method that is being explored. It may be effectively employed to reduce the amount of viral exposures occurring, especially in high incidence areas. This might be effectively utilized to stem the current pandemic. Vaccines of this nature are under current investigation (7, 87, 107, 151).

Therapeutic molecules that target viral processes required for the viral life cycle are the most effective current means of inhibiting HIV related disease. These agents do not prevent infection, but inhibit viral replication and thus prolong the period from infection to the development of AIDS. Agents that target viral proteins have the potential to cause minimal detrimental effects on uninfected cells of the host. These agents do not eliminate the infection from the host, but rather control or reduce

the viral load to a level that is contained by the immune system. Because HIV infects the cells of the immune system, utilizing these therapeutic agents early spare the cells necessary to contain the virus. However because HIV is so capable of change, mutations inevitably arise, allowing variants to escape inhibition, therefore allowing progression towards AIDS (32, 65). Resistant HIV variants are known to exist for every anti-retroviral agent in current use; therefore further research in anti-retroviral compounds continues to be important (48, 98, 162).

The majority of antiretroviral compounds in use today act by targeting well-defined viral enzymatic processes, including Reverse Transcriptase, Protease, and Integrase. Recently new classes of compounds have emerged that target protein-protein interactions and interfere with processes like viral fusion and entry (47, 121). When used as a single therapy, all of the drugs discussed result in the rapid selection of resistant HIV variants. Drug combinations, especially 3 or more with varying activities, are key to successful long-term therapy of HIV infected patients (172, 189).

Viral Proteins and Therapeutics

Reverse Transcriptase

Reverse Transcriptase (RT) was originally discovered in 1970 (Baltimore, Temin, and Mizutami), and is important for the formation of current models of evolution, and the concept of the “RNA to DNA world”.

The HIV Reverse Transcriptase enzyme is encoded in the *pol* gene. As the viral particle buds and matures, RT assembles into it as part of the Gag-Pol polyprotein precursor. Mature RT is cleaved from the precursor by the viral Protease, and ends up in the interior core of the particle. HIV-1 RT exists as a heterodimer containing p66 and p51 subunits, which perform the complex task of reverse transcription. The p66 subunit contains both the DNA Polymerase and RNAse H activities, whereas the p55 lacks the RNAse H domain. RT is a DNA polymerase that importantly lacks a proofreading function, since it does not contain 3' exonuclease activity that could excise mispaired nucleotides. This property accounts in part for the high HIV mutation rate. The structure of HIV-1 RT polymerase domain, like most other DNA and RNA polymerases, has been compared to a right hand with thumb, palm, and fingers making up separate domains. Several Asp residues in the active site are highly conserved between HIV isolates (92, 115).

Reverse Transcriptase Inhibitors

Nucleoside Analogs

Anti-cancer agents targeted to polymerases were shown early in the HIV pandemic to have effectiveness against HIV Reverse Transcriptase. For example zidovudine (AZT), didanosine (DDI), zalcitabine (DDC), stavudine (D4T), and lamivudine (3TC) were all used initially as cancer chemotherapeutics. The compounds are nucleoside

analogs converted to nucleotides within the host cell by phosphorylation, and most often function as substrate competitive inhibitors. When these nucleotide analogs are incorporated into DNA, polymerization is chain terminated. Toxicity is unfortunately a problem because these compounds can also interfere with mammalian DNA polymerases. And of course HIV can quickly develop resistance when these compounds are used as single therapies.

Nucleoside analog resistance is well documented at specific amino acid sites within the Reverse Transcriptase enzyme (33, 57, 162). Most mutations are in areas of enzyme template interaction (Amino Acids 41, 67, 69, 70, 74, 215, 219). These mutations augment the ability of HIV-1 RT to excise chain terminating nucleoside inhibitor monophosphates from the previously terminated DNA chain. Other resistance mutations are close to the enzymes active site (amino acid 184), or directly affect how the enzyme interacts with NTPs (amino acids 65, 70, 74). These mutations can increase the selectivity of Reverse Transcriptase for the incorporation of natural dNTP substrates over the nucleoside inhibitor dNTPs.

Nonnucleoside Analogs

Other compounds have been identified that inhibit Reverse Transcriptase (RT) in a noncompetitive manner. They are structurally diverse and do not require metabolic activation. They are also less toxic than the nucleoside analogs. These inhibitors occupy a binding pocket

located proximal to the RT active site and allosterically cause inhibition. Each inhibitor occupies the site in a unique way that is dependent on its structure. All of the inhibitors affect the rate of reaction catalyzed by Reverse Transcriptase, and inhibit enzyme structural flexibility (24, 43, 141, 152).

Nonnucleoside Analogs readily generate resistance by selection for mutations in amino acids of the viral Reverse Transcriptase enzyme (amino acids 98 to 108, 138, 179, 181, 188, 190, 236). These mutations prevent the inhibitor from binding into the Nonnucleoside Reverse Transcriptase Inhibitor pocket. Some primary isolates of HIV-1 and HIV-2 are naturally resistant to these compounds.

Protease

Protease is another viral enzyme translated from the *pol* gene. It functions during the viral assembly process to cleave the Gag and Gag-Pol precursors into their mature protein products. Sequence comparisons of Protease enzymes from diverse retroviruses show a striking relationship to cellular aspartic proteases that use two apposed Asp residues in their active sites. Examples of mammalian aspartic proteases include pepsin and renin. HIV-1 Protease exists as a homodimer and has a conserved structure. Dimerization is crucial for the activity of the enzyme, with each subunit of the dimer contributing an Asp residue to the active site.

Protease recognizes hydrophobic peptide sequences eight amino acids in length with two-fold symmetry around the cleavage site (27, 92).

Protease Inhibitors

Protease inhibitors are typically peptide cleavage substrate mimics that compete for the viral enzyme with the polypeptide precursor substrates. Although these inhibitors have diverse chemical forms, many form similar conformations in the substrate-binding cleft of the Protease homodimer. Others function by preventing the dimerization of the enzyme, therefore preventing activity (1, 14, 163).

Protease inhibitors also generate resistant variants, although after a longer time frame than Reverse Transcriptase inhibitors. Mutations in the Protease enzyme active site, or compensatory areas that help accommodate active site changes, result in resistance (amino acids 10, 20, 24, 46, 54, 63, 64, 71, 82, 84, 90). Usually three or more amino acid substitutions are required for resistance to occur (98, 126, 148, 162, 164).

Integrase

Integrase is another viral enzyme encoded by the *pol* gene. The viral Integrase enzyme allows for integration of the reverse transcribed double stranded viral DNA into the host genome. Integrase contains three known domains that are highly conserved: A zinc finger HHCC domain, a catalytic core DDE domain, and a C terminal DNA binding domain. The

HHCC domain, in the presence of zinc, promotes the assembly of integrase dimers into tetramers. This form is necessary for formation of a stable complex with the viral DNA ends. The enzyme performs two main functions: endonucleolytic removal of 3' terminal dinucleotides from the LTR of each viral DNA strand, and the viral DNA strand transfer into the genomic DNA (4, 171). Integrase has also been shown to structurally interact with Reverse Transcriptase. The importance of this interaction is still under investigation (179).

Integrase inhibitors

Although only in the clinical trial stages, Integrase inhibitors are some of the newest anti-HIV enzymatic inhibitors being developed. The compounds either interfere with the zinc finger area that is associated with DNA cleavage, or the enzyme catalytic core that inhibits the strand transfer step of integration (179). Very little is known presently about clinical resistance patterns to these compounds, even though resistance has been shown in vitro (13, 82, 105). Interestingly some Reverse Transcriptase inhibitors have been shown to interfere with Integrase. How these compounds could be cooperatively employed against HIV is still under investigation (124, 125).

HIV-1 Envelope Protein

HIV-1 entry into host cells is mediated by the viral envelope proteins gp120 and gp41. These glycoproteins are arranged on the surface of the viral particle in trimeric complexes containing three gp120-gp41 heterodimers. (56). These structures form shortly after translation in the endoplasmic reticulum, and traffic to the plasma membrane becoming part of the viral membrane during budding. To mediate viral entry gp120 binds first to the host CD4 receptor. This induces a conformational change in gp120 that allows interaction with the chemokine coreceptor (CXCR4 or CCR5). HIV-1 utilizes CCR5 early in disease progression, but later switches in about 50% of human cases to CXCR4 utilization (123).

Coreceptor usage is one area of investigation for AIDS pathogenesis. It is interesting that it occurs around the same time as the dramatic drop in CD4+ T cells preceding AIDS, but whether this is a cause or effect is not clear. This phenomenon is currently being studied in the macaque model, as well as HIV infected human cohorts (37, 186).

Coreceptor usage is clearly regulated by the Env protein. Sub domains in gp120 V3 loop have been identified that affect the tropism for CCR5 or CXCR5. These domains interact with different areas in the chemokine receptors. CCR5 tropic Env interacts with the N terminus of CCR5 using the base of the V3 gp120 loop, whereas CXCR4 tropic Env interacts with the extra cellular loops of CXCR4 using the gp120 V3 flexible stem (16, 130). HIV and the other primate Lentiviruses differ from other retroviruses in the requirement for a second cell surface molecule for

infection. The primary receptor CD4 is sufficient for attachment of the virus to the host cell surface, but not for viral entry. Interaction with a second co receptor initiates viral fusion with the host cell membrane (123).

The mechanism of viral envelope protein mediated membrane fusion is well understood. It involves the viral envelope protein gp41. The N terminus of gp41 contains a fusion peptide that becomes activated after CD4 and coreceptor binding. This peptide inserts into the target cell membrane. gp41 remains anchored to the viral membrane by a transmembrane region at its C terminus. There are two helical regions within gp41 that bind to each other and bring the N and C terminal regions into close proximity causing the host and viral membranes to fuse. These helical regions are called heptad repeat region 1 (HR1) and heptad repeat region 2 (HR2). They function in the context of the gp41 trimer. When they interact, a six-helix bundle is formed that overcomes the forces separating the membranes allowing for hemi-fusion and finally fusion. Fusion of the membranes forms a pore that allows for particle release into the host cell cytoplasm. Certain amino acids in gp41 especially in the HR1 domain have been shown to influence fusion capacity of the Env protein trimer (95, 117).

CCR5 Inhibitors

The interaction between HIV-1 gp120 and the coreceptor CCR5 occurs early in infection, and is required for viral entry into the host cell.

Natural occurring CCR5 antibodies have been isolated that are protective against epithelial cell infection with HIV-1 (23). Compounds that bind to CCR5 and create allosteric changes in the receptor have been developed (maraviroc, vicriviroc) (142). These molecules potently prevent the entry of CCR5 tropic HIV-1 in vitro, and are currently being studied in Phase II and III human trials. Because CCR5 is a chemokine receptor used for normal body functions, there is concern of potential adverse side effects. Currently the available data suggests that the compounds seem to have had no adverse effects on the immune system or organ function. Some reports of malignancy have been reported, but there has been no causal relationship yet established. Another concern with the inhibitors is the selection of CXCR4 tropic virus, which might be more pathogenic. It has already been demonstrated that these compounds have no effect against CXCR4 tropic virus, but CXCR4 inhibitor compounds are also in development (8, 52, 147).

Resistance to CCR5 compounds is mediated by the development of changes in gp120 that allow interaction to occur with CCR5, despite CCR5 being bound to the inhibitor. Subdomains in gp120 V3 have been identified that selectively modulate the sensitivity to the chemokine coreceptor inhibitors (133, 142, 187).

Viral Fusion Inhibitor Enfuvirtide (T-20)

Molecules that inhibit viral fusion have been developed. Enfuvirtide (T-20) is a peptide fusion inhibitor now in clinical use. This compound has potent antiviral activity against HIV-1 in vivo and has demonstrated significant benefits when used in combination with other anti-retroviral compounds. Enfuvirtide is a competitive inhibitor derived from the peptide sequence of HR2. Resistant variants to enfuvirtide arise as early as 2 weeks when used as a single therapy. The majority of enfuvirtide resistance mutations have been reported in gp41 amino acids 36-45 in HR1. This also corresponds to stem loop IIC and III of the Rev Responsive Element (119, 136, 156, 173). Presumably these mutations work by allowing HR1 to bind to HR2 more readily than it binds to the T-20 peptide. Enfuvirtide resistance can also arise by increased envelope expression or production in the viral variants. Secondary resistance mutations have been identified in gp120, often in the V3 loop, that affect Env interaction with its chemokine coreceptor (29, 48, 49, 101, 135). Mutations that affect coreceptor engagement and membrane fusion rates can alter sensitivity to entry inhibitors (135).

Figure 2 illustrates the functional domains and amino acid sequence of gp41 with the HR1 and HR2 regions, and its relationship to underlying RNA sequences, which forms the Rev Responsive Element. As discussed Enfuvirtide (T-20) is derived from the HR2 region and functions as a competitive inhibitor of six-helix bundle formation by preventing the association of the heptad repeats in gp41.

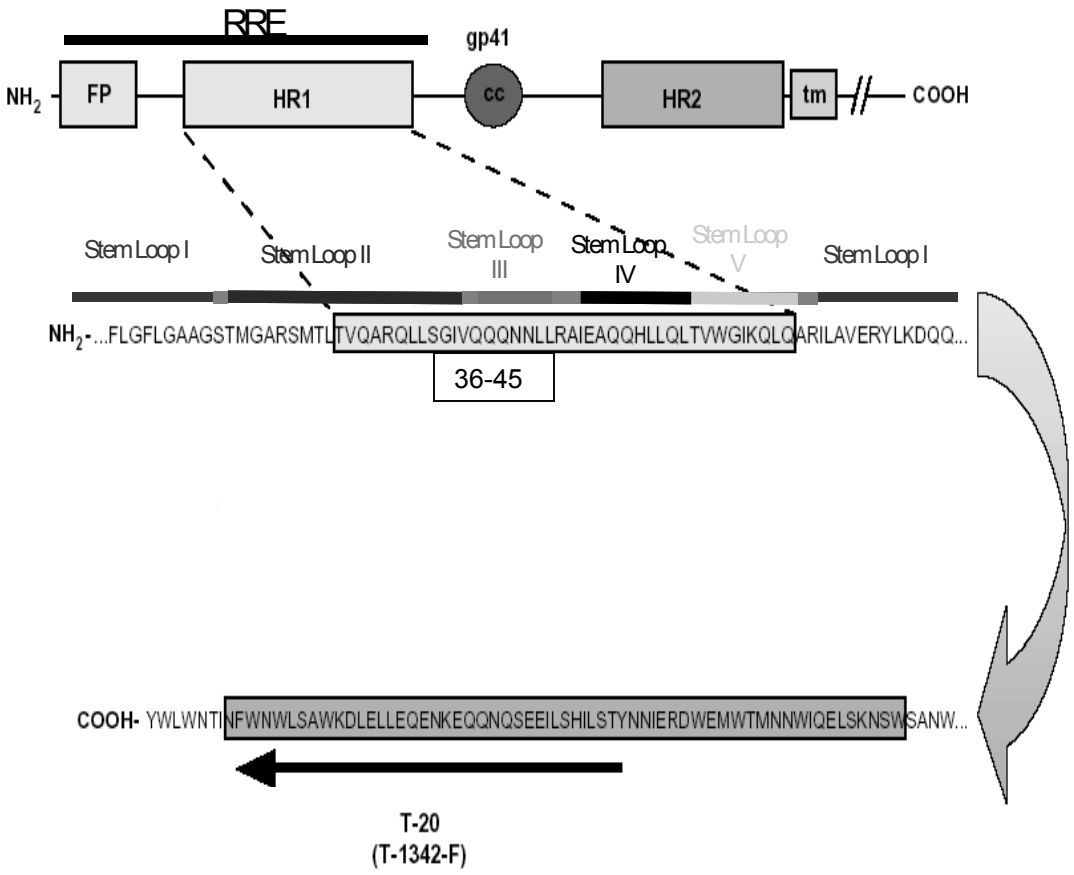


Figure 2. T-20 and gp41.

An illustration of Env gp41 is shown with the functional domains and location of the RRE annotated. HR1 and HR2 are shown with the corresponding amino acid sequence and location of the stem loops of the RRE illustrated. T-20 is derived from HR2 as illustrated. Changes in gp41 amino acids 36-45 (GIVQQQNNLL) make up over 90% of the known T-20 resistance mutations.

Tat

Tat is a viral regulatory protein that interacts with a RNA stem loop element in the 5' R region of the viral mRNA (TAR). This interaction puts Tat in proximity to associate with the cellular transcriptional machinery, allowing the protein to augment the transcription process (25, 181). Tat has been shown to recruit p-TEFb, comprised of cyclin T1 and cdk9, that allows for the phosphorylation of the C Terminal Domain (CTD) of RNA Polymerase II thereby increasing processivity. Tat is a potent trans activator, enhancing transcription of viral messages thousands of fold. Tat is translated from fully spliced viral mRNA derived from two exons within the central region and *env* gene of the viral genome. The *tat* gene is necessary for normal viral replication. Mutations in *tat* have resulted in latently infected cells that maintain the HIV-1 provirus. If these cells are induced with transcriptional activators, such as TNF-alpha (which induces NFKB), viral replication can be resumed (2, 53, 94). Mutations in the TAR region also affect Tat trans activation.

Tat inhibitors

Peptide molecules, Tat decoys, that inhibit the interaction of Tat with the Tar have been described (10). An inhibitor developed by Hoffmann-LaRoche (Roche) worked well in vitro, but failed in patients. Industry by and large has abandoned efforts to develop Tat inhibitors. But research still continues on this important viral protein, and the potential for targeting Tat with an effective therapeutic remains.

Nef

The Nef protein primarily functions for viral immune evasion and modulation. The Nef protein in HIV differs from that in SIV. In wild Sooty Mangabey Monkeys, SIV does not produce AIDS. Although the virus produces the same CD4+ T cell depletion as HIV in the early stages of infection, it fails to promote immune activation in the mucosa in the presence of fewer CD4+ cells, which is protective. The infection does not progress to systemic CD4+ T cell depletion and AIDS. Nef is one of the factors thought to contribute to the differences in pathogenicity of these viruses. SIV with Nef deletions also fail to produce AIDS in studies performed in Macaques (50, 93, 146). In HIV Nef is considered one of the contributory factors to the development of AIDS. Cohorts studied of long term nonprogressors (HIV infected individuals failing to progress to AIDS) have demonstrated mutations in Nef (5, 54, 55, 86).

Several functions have been attributed to Nef from studies using in vitro culture systems. Nef can regulate the expression of cell surface molecules used for entry by the virus and antigen presentation by the host cell. For example Nef down modulates surface molecules such as CCR5, CXCR4 (Chemokine coreceptors for HIV), CD4 (MHC1 receptor for HIV), MHCII (antigen presentation), CD28 (Immune stimulation), and DC sign (Dendritic receptor for HIV) by either degradation in lysosomes or sequestration in the Trans Golgi. This prevents superinfection of cells with

other HIV viral particles, and reduces the immune systems ability to recognize infected cells (157, 169, 174).

Nef also seems to regulate immune products having a positive effect on the viral infection. Nef induces CD40 signaling that recruits and activates immune cells rendering them susceptible to infection. Nef also suppresses Ig class switching in B cells preventing the production of appropriate neutralizing antibodies (129). Additionally it has been reported that Nef regulates cell surface molecules, protecting infected cells from detection and destruction. Nef seems to cause selective apoptosis by up regulation of FasL on uninfected bystander CTLs while protecting those already infected with virus. Nef down regulates ligands (MICA ULB1/2) of activation receptors NKG2D and inhibits natural killer cell mediated toxicity of infected cells. Nef also causes a differential expression of HLA causing a down regulation of the antigen presenting HLA A/B to CTL while maintaining HLA C/E that prevent NK lysis (3, 30, 36, 100, 122). The problem has been associating the effects of nef in vitro with the biologically relevant roles of the protein in human pathogenesis.

No current inhibitors exist for this protein. Nef is not required in tissue culture for replication of HIV. The importance of this protein relates to how the virus interacts with the human host and functions in vivo. This protein is clearly important in HIV pathogenesis but its exact role has remained elusive.

Vif

Vif is another viral accessory protein made from a singly spliced mRNA. Vif is nonessential in certain cell types, but in others it provides an essential function for the virus. We know that certain cell types are non permissive for HIV-1 that are Vif (-). These cells express APOBEC3G, which is able to alter the genetic makeup of the infecting HIV. APOBEC3G is a cytosine deaminase that acts on HIV-1 during reverse transcription of the first DNA strand (minus strand). By causing C to change to U (recognized as T), an A is incorporated in the second viral DNA strand (plus strand) rather than a G. Thus APOBEC3G causes G to A mutations that can be detrimental to the viral genome. APOBEC3G is incorporated into the viral particles, and exerts the cytosine deamination during reverse transcription, after infection of another cell.

Vif counteracts APOBEC3G function and reduces viral hypermutation (170). To do this Vif binds directly to APOBEC3G and triggers the polyubiquitination and proteasomal degradation of this protein before it is incorporated into the viral particle. Similar molecules, APOBEC3F and 3B, also cause cytosine deamination but are resistant to Vif. A significant amount of current HIV research focuses on APOBEC3G and Vif as potentially novel viral drug targets (80, 137). However no actual compounds targeting Vif are under development.

Vpr

The viral accessory protein Vpr is not essential for HIV replication in most cell types. Vpr has been shown to assist the viral preintegration complex into the nucleus of the host cell. The C Terminus of Vpr can bind DNA and recruit endonucleases to create double strand DNA breaks, thus assisting in integration of the viral genome (112, 160).

Vpr also seems to alter the normal cell creating conditions that allow for optimum viral replication and particle production. Vpr causes G2/M cell cycle arrest preventing mitosis (103). This state of arrest seems to promote virus production. It has also been suggested that Vpr interacts with proteins involved in transcription to regulate replication of the viral genome, and it also induces host cell cytotoxicity due to induction of apoptosis through the ATM/ATR checkpoints (118, 166).

Immune modulating effects of Vpr have also been reported sometimes in combination with Nef. Vpr may affect immune cell differentiation, activation, antigen presentation, and cytokine release. If has been reported that Vpr down regulates stimulatory molecules such as CD28 and INF, and up regulates suppressive molecules such as CTLA-4 perpetuating viral infection of the host (40, 103, 104, 118, 143, 160, 167).

Because this protein is not essential for the virus, the impact anti-Vpr therapies would exert to prevent AIDS is uncertain. No anti-Vpr therapies are currently under development.

Vpu

Vpu, another nonessential viral accessory protein, has been shown to play a function in viral budding. Vpu promotes viral release by interaction with the viral matrix protein (120). Vpu also prevents particle endocytosis during budding by down regulation of the receptor CD4 on the host cell membrane. Env protein sequesters the CD4 molecules in the ER, and then Vpu targets the molecules for ubiquitin dependent degradation in the proteasome (6, 77, 89, 110, 120, 165, 174). Compounds that inhibit Vpu function have yet to be developed, and since the protein is nonessential for virus replication, it is not clear what impact such compounds would have as therapeutics.

Rev and the Rev-RRE pathway

The Rev protein, and the RNA export it mediates, are essential for replication of HIV-1. The Rev-RRE pathway is necessary for transport of unspliced and incompletely spliced viral mRNAs, as well as viral genomic RNA, from the host cell nucleus to the cytoplasm (46). Proteins encoded by these intron-containing mRNAs (Gag/Pol, Vif, Vpu, Vpr, Env) require Rev-RRE for their expression. However proteins encoded by fully spliced mRNAs (Tat, Rev, Nef), are expressed by normal cellular mechanisms.

Rev is a 116 amino acid protein containing several functional domains (131), and mutations in each of these defined domains abolish viral replication (39, 42, 91, 154, 161). Two of the domains consist of nuclear localization and export signals that allow the protein to shuttle in

and out of the nucleus. The domain containing an arginine rich nuclear localization signal (NLS) is also the part of the protein that mediates the specific binding of Rev to the Rev Responsive Element (RRE). On either side of the NLS Rev contains domains that allow it to multimerize (44). Rev multimerization on the RRE masks the NLS of the protein (42, 69).

The RRE is a 234 nucleotide multiple stem loop RNA structure (51, 58, 84, 177) that is located in the *env* coding region (Figure 3). The whole RRE structure is highly conserved between HIV isolates and is very complex, containing multiple stem loops that are likely to have some important function in the Rev-RRE pathway. RNA footprinting studies of the RRE indicate that a U loop and G loop of stem loop I, and the majority of stem loop II (A, B, C) show Rev protection suggesting Rev multimerization in these areas (97). Stem loops III, IV, and V have not been shown to be essential for Rev binding and their overall importance to Rev-RRE function is largely unknown. Stem loop IIA is highly conserved among HIV-1 sequences and appears to be important to direct Rev binding to the high-affinity binding site of stem loop IIB.

The RRE contains at least one high affinity binding site for Rev in stem loop IIB. Modeling and NMR studies have shown that stem loop IIB forms a major groove with purine-purine pairings. It can be stabilized by a peptide derived from the Rev NLS helix (59, 83, 85, 88, 97, 111, 176). The Rev high affinity interaction with the Rev Responsive Element has been well defined, but less is known about how the protein multimerizes on this

structure, about other regions of the RRE, or how the Rev-RRE complex structurally interacts with cellular proteins (28, 90, 114). Drug development efforts have concentrated on disrupting the high affinity interaction of Rev with the RRE stem loop IIB (76). Figure 3 illustrates the NL4-3 RRE structure that was recently solved using SHAPE methodology by our collaborator Chris Badorrek. Two primary structures are published for the NL4-3 RRE. One completely separates III and IV into separate stem loops. The other combines the III and IV stem loops, but differs from Figure 3 slightly in the nucleotides comprising the stem loop III and IV regions (76, 175).

The current model of Rev mediated export visualizes RNA as "cargo" bound to several molecules of Rev. The nuclear export signal (NES) of Rev binds the cellular exportin Crm1. This complex also binds RanGTP for targeting to the nuclear pore complex (9, 62, 96). The Rev-RRE-Crm1-RanGTP complex is thought to associate with other cellular proteins, such as helicases (99, 182) and nuclear porins, as it translocates to the cytoplasm, but the exact role of these factors is still under investigation (17, 46, 154, 168). For example, mutants of cellular protein hRIP, a possible nucleoporin protein, cause the Rev-RRE mRNA complexes to localize in the nuclear periphery (183). Interestingly the Crm1 export pathway used by Rev is generally thought to transport cellular proteins, not RNA; but a small subset of RNA also seem to utilize Crm1 (67, 168).

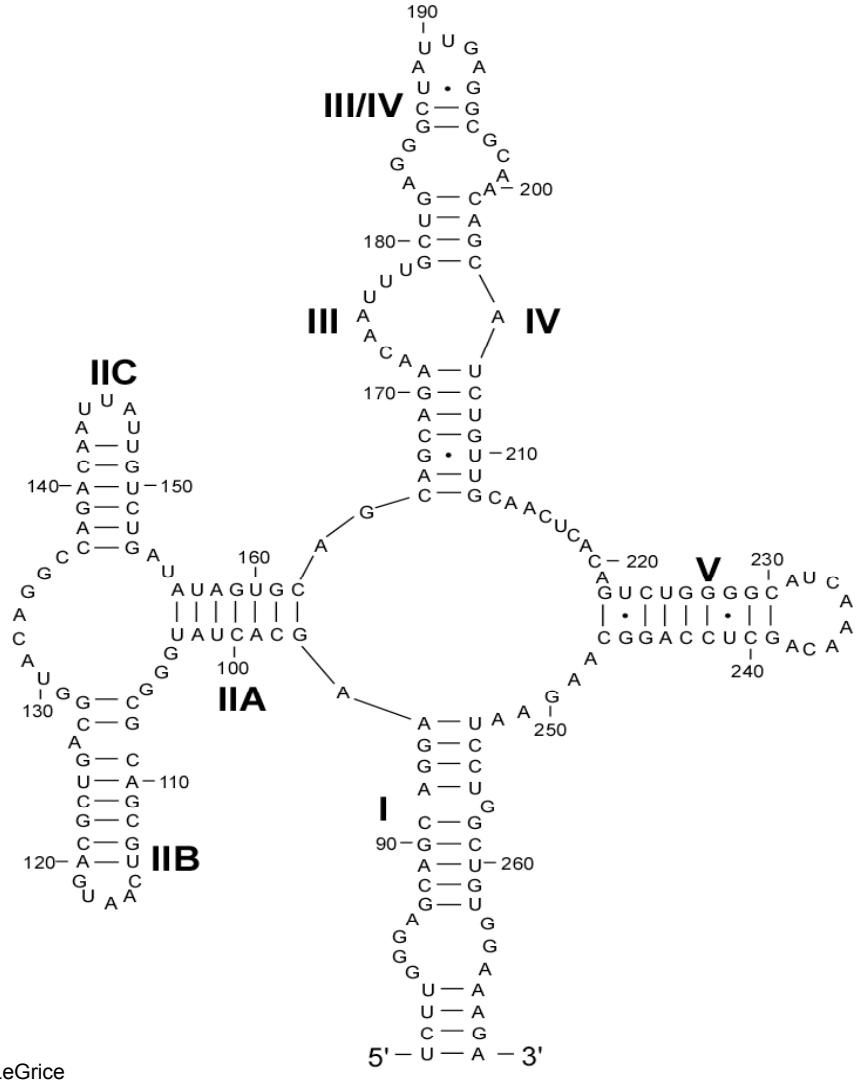


Figure 3. Structure of NL4-3 Rev Responsive Element.

The RRE contains five stem loops with the high affinity binding site of Rev located in stem loop IIB. Figure illustrates only part of stem loop I.

Research on other retroviruses, such as EIAV, HTLV, and MMTV, has revealed proteins that are functionally equivalent to HIV Rev (19, 45, 109). The Rev homolog in Human T-Cell Leukemia Virus Type-1 (HTLV-1), Rex, does not multimerize with Rev in vivo, but can bind to the HIV-1 RRE. Rex however was shown to preferentially bind to stem loop IV and V of the RRE. They are functionally equivalent for their respective viruses, and both utilize the Crm-1 export pathway (20-22).

Studies of simpler retroviruses discovered an entirely separate pathway for the export of their intron containing mRNAs. Mason Pfizer Monkey Virus (MPMV) uses another RNA element, the Constituative Transport Element (CTE), in a cellular mRNA transport pathway requiring Tap/NXT1 (21, 26, 158). The CTE is located near the 3' terminus of the MPMV mRNA and has a structure unique to the RRE. One could infer that more complex retroviruses developed a pathway that was less dependent on the host cell for viral mRNA export.

Figure 4 illustrates the open reading frames of HIV, the location of the RRE, and the three main size and splicing categories of HIV mRNA compared to the two of the simpler retrovirus Mason Pfizer Monkey Virus. The location of the MPMV CTE is demonstrated near the 3' LTR. The Figure does not illustrate the complex alternative splicing patterns and mRNA products actually produced by the viruses.

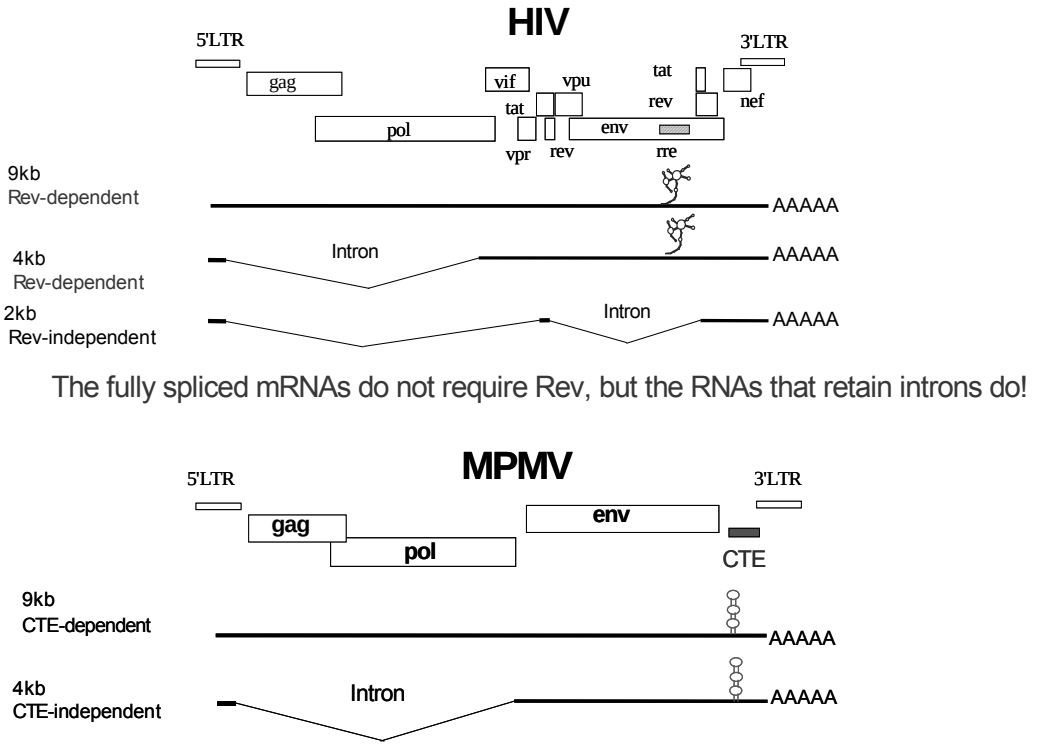


Figure 4. HIV and MPMV Open Reading Frames and mRNAs.

HIV and MPMV both produce intron containing mRNA. These species require RNA elements located in the messages for transport from the host cell nucleus to the cytoplasm. HIV has adapted a viral protein to associate with the RRE for this process. The majority of the viral messages require the Rev-RRE pathway for expression.

Rev prevents the degradation of intron containing messages in the nucleus that have not undergone full splicing and promotes their export, but how Rev interacts with the cellular splicing machinery is still under investigation. Exon splicing enhancers and silencers within the HIV genome have been identified that affect the expression of HIV-1 genes (35, 132). Rev activity also seems to be affected by genomic signals, AU rich INS elements in *gag*, *pol*, and *env* genes (73, 188). The Rev-RRE pathway has been demonstrated to affect the level of translation of viral messages, as well as associate with eIF5A (127). Rev also is thought to increase effective polysome loading, and increase the polyAP-1 protein association with RRE containing RNA (41).

The Rev-RRE pathway is a target of clinical relevance. RRE, *rev* mutations are present in people infected with HIV. Studies of both naïve and HAART treated patients have documented that the progression to AIDS is slowed with reduced Rev-RRE function (18, 128). RRE, *rev* mutations have also been found to exist in HAART treated patients with discordant viral load to CD4 counts (CD4 counts remaining high despite high viral loads) (145). The selection of viral quasi species containing these mutations can affect the clinical outcome of HIV infected patients. Because Rev is essential for viral replication, and because defects in *rev* and the RRE have already demonstrated importance towards disease course, the Rev-RRE pathway presents an excellent target for future anti-retroviral therapy.

Rev-RRE Small Molecule Inhibitors

Small molecule compounds that inhibit HIV-1 replication and Rev-RRE function have been previously identified (113). An aminoglycoside antibiotic (neomycin), previously known to inhibit structures in ribosomal RNAs, has been shown to inhibit Rev function by binding to the RRE and inhibiting Rev-RRE interaction (102, 184). Leptomycin B has been shown to target Crm1, preventing the NPC transport of Rev-RRE mRNA. Both these compounds are too toxic to use as systemic therapies for HIV. Various peptides and binding mimics are also under preliminary investigation for Rev inhibition (74, 78, 140, 159). Heterocyclic compounds, such as diphenylfuran cations, have been described that prevent HIV replication by Rev-RRE interaction inhibition (178, 185). However none of the mentioned compounds have yet been developed for current clinical use. The pharmaceutical industry has not pursued in earnest this HIV target.

Clinical Therapy for AIDS

HAART Therapy continues to be the most effective treatment of HIV-1 infected people and AIDS patients. HAART stands for Highly Active Antiretroviral Therapy and consists of treatment with combinations of anti-retroviral compounds (12). HAART employs the understanding that the virus must be targeted in multiple essential processes to achieve adequate suppression to abate disease and reduce the incidence of

resistance. Newer therapies like those that combat viral fusion are being added to HAART regimens with success.

Multiple Drug Resistance

Despite the advent of HAART HIV-1 has also been able to produce variants with multiple drug resistance mutations. Reverse Transcriptase and Protease mutations that confer drug resistance can impair the capacity of HIV to replicate. However these less fit multi drug resistant (MDR) viruses can still establish persistent infections as dominant quasi species under selective pressure (81). If the drug selective pressure is removed, drug susceptible variants can rapidly outgrow the drug resistant viral quasi species in vivo. Dual growth selection studies have shown the level of MDR virus decreases compared to wild type virus in the absence of drugs, but when selective pressure is reapplied the same mutations can be rapidly expressed. It has been observed however that some combined mutations (multiple *pol*) can increase the fitness of the resistant variant over wild type in the absence of drug pressure (47, 48, 98, 162). This increased fitness has also been observed with some Enfuvirtide resistance mutations (95). HIV is highly adaptive and unfortunately is able to persistently infect the human host despite anti-retroviral therapy.

Because of the adaptive nature of HIV, there have been increased efforts to tailor anti-retroviral therapy based on patient viral genotyping, and known viral drug resistance patterns. Databases of primary

resistance mutations in *pol* affecting Reverse Transcriptase and Protease have been useful in predicting clinical resistance based solely on genotype (126). Enfuvirtide mutations are more difficult to predict based on genotype alone. Resistance can be due to multiple factors and reflect the complexity and dynamic nature of *env*. Even more interesting to this research is the overlap of the RRE with gp41 *env*. Resistance mutations resulting in competent virus would need to conserve both the RRE and gp41 functions (119). It would be interesting to know if this constraint could increase the effectiveness of combinations of viral fusion and Rev-RRE inhibitors. Rev-RRE inhibitors and Fusion inhibitors could be used in combination as “convergent” therapies. Rev-RRE inhibitors may also be utilized as salvage therapies for Fusion inhibitor resistance. As the practice of tailoring therapy to the patient HIV genotype continues to expand, knowledge of resistance mutations becomes increasingly important.

Chapter 2

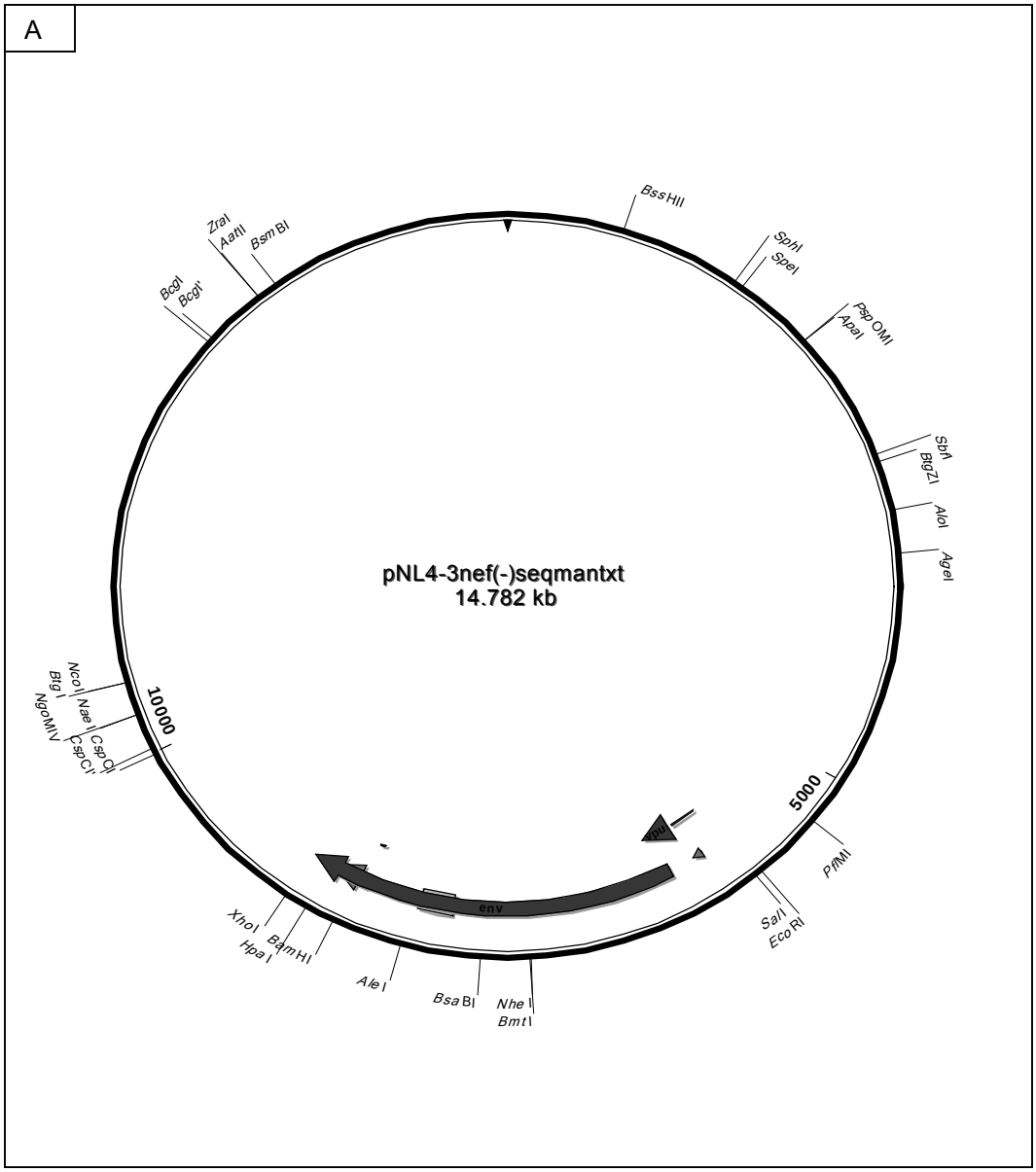
Material and Methods

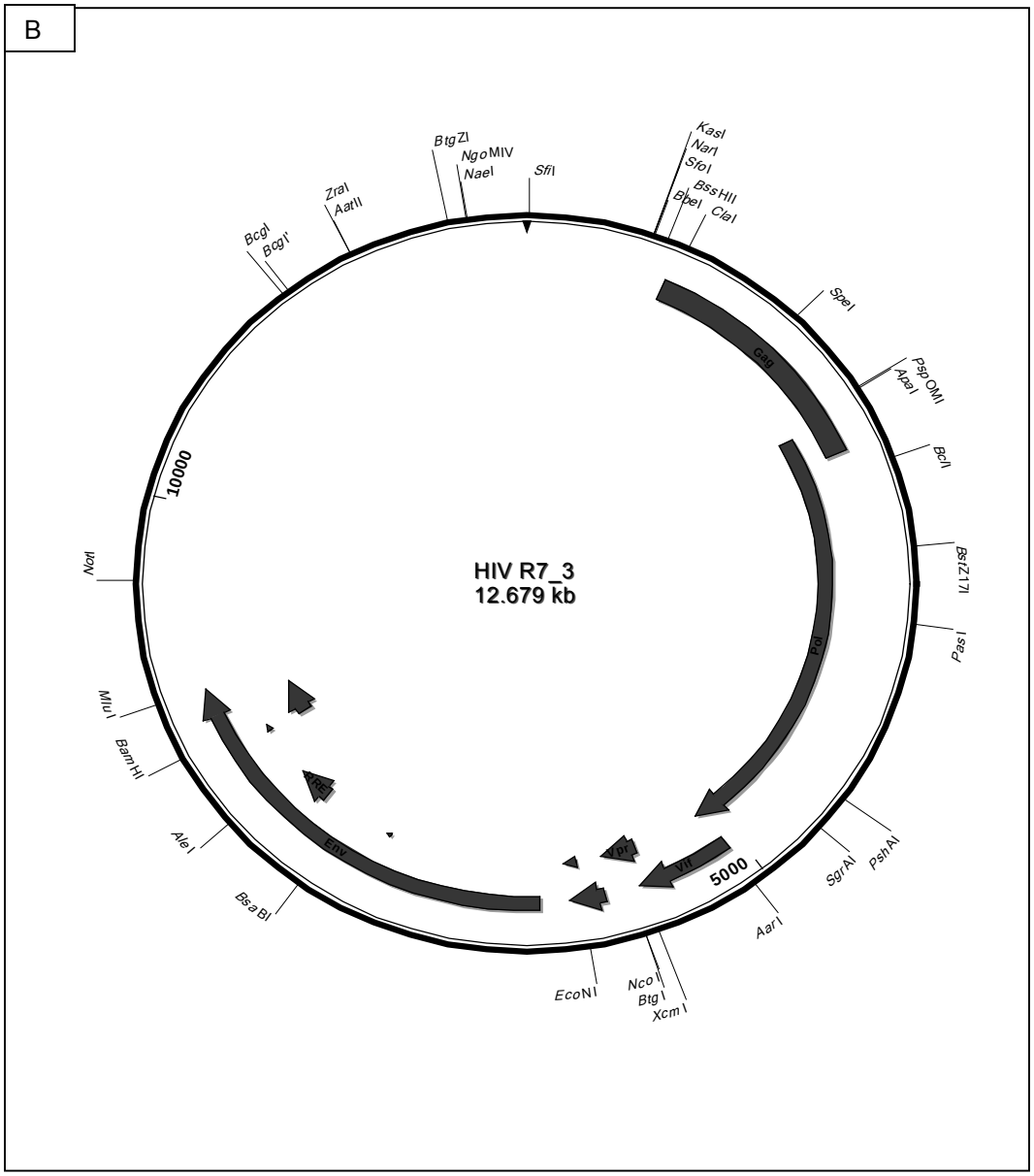
Viral Strains Studied

NL4-3 and HXB-2

Our research utilizes HIV-1 Clade B viruses with differing genetic backgrounds. NL4-3 (Genbank Accession numbers M19921 and AF324493) represents the standard laboratory strain of HIV-1 Clade B viruses containing all the genes identified: *gag, pol, env, tat, rev, nef, vif, vpr, vpu* (89, 170). It is a recombinant virus with the 3' end originating from the NY5 strain (Genbank Accession number M38431), and the 5' end from the BRU strain (Genbank Accession number KO2013-also known as LAV-1) of HIV. The HXB2 (Genbank Accession number KO3455) HIV-1 virus, and its derivative R73 virus, contains several protein deletions compared to NL4-3. HXB-2 contains a T insert in *vpr* causing a frameshift, the *vpu* start codon is changed to ACG coding for Threonine, *tat* has a premature stop, and *nef* contains a premature stop and deletions. Plasmid Diagrams of NL4-3 nef (-) and R73 illustrated in Figure 5.

Figure 5. A: Plasmid Diagram of NL4-3 nef (-). B: Plasmid Diagram of R73 Unique restriction enzyme sites annotated.




B
HIV R7_3
12.679 kb
10000
5000
Gag
Pol
Vif
Vpr
Tat
Rev
Env
Nef
KasI
NarI
SfoI
BssHII
BbeI
ClaI
SpeI
PspOMI
ApaI
BclI
BstZ17I
PasI
PshAI
SgrAI
AarI
NcoI
BtgI
XcmI
EcoNI
BsaBI
AleI
BamHI
MluI
NotI
BcgI
BcgI'
ZraI
AatII
BtgZI
NgoMIV
NaeI
SfiI

Plasmids and proviral constructs

Number in parenthesis denotes HamRek laboratory internal archive number.

Nucleotide sites refer to NL4-3 numbering system, except for R73 viral clones that refers to R73 numbering system.

1. pNL4-3 (#1145)- infectious proviral clone of HIV-1 constructed using the 5' end of the NY5 HIV-1 isolate and the 3'end of the LAV-1 isolate (Reference Genbank Accession number AF324493)
2. pNL4-3 rev (-) (#1146)-#1145 with a PCR change to 1st exon of Rev, Rev aa 23 TAT→TAA, inducing a premature stop
3. pNL4-3 nef (-) (#1272)-#1145 with a 150bp deletion in nef moving the XhoI unique restriction site just downstream of the nef ATG start codon
4. pSPRRE (#1627)- pSP64 vector with NheI to BamHI insert containing the NL4-3 RRE
5. pNL4-3 nef (-) RRE61 (#1721)-#1272 with NheI to BamHI from Clone 61 RevM10 mutant with G→A mutations at RRE sites 7864 and 7945
6. pNL4-3 nef (-) 1.3 (#3536)-#1272 with NheI to XhoI from Clone 1.3 Rev-RRE inhibitor mutant with A→G mutations at RRE sites 7854 and 7936

7. pNL4-3 nef (-) 5.7 (#3537)-#1272 with NheI to XhoI from Clone 5.7 Rev-RRE inhibitor mutant with A→G mutation at RRE site 7936, A→C mutation at env site 8091, and T→C mutation at env site 8095
8. pNL4-3 nef (-) 9.3 (#3538)-#1272 with NheI to XhoI from Clone 9.3 Rev-RRE inhibitor mutant with A→G mutation at RRE site 7854, A→G mutation at RRE site 7936, G→A mutation at env site 8184, and C→T mutation at env site 8773
9. pNL4-3 nef (-) 13.10 (#3539)-#1272 with NheI to XhoI from Clone 13.10 Rev-RRE inhibitor mutant with A→G mutation at RRE site 7854
10. pUC Rev (#3542)- pUC vector with NL4-3 Rev amino acid coding sequence
11. pNL4-3 nef (-) peak 7 (#3543)-#1272 with NheI to BamHI from #3560 with RRE mutation C→T at site 7836 identified in peak 7 103833 resistant variant
12. pNL4-3 nef (-) single mutagenized peak 7 (#3544)-#1272 with NheI to BamHI from mutagenized #3560 with mutation C→T at site 7836 and additional mutation C→A at RRE site 7837
13. pNL4-3 nef (-) double mutagenized peak 7 (#3545)-#1272 with NheI to BamHI from mutagenized #3560 with mutation C→T at site 7836 and additional mutations C→A at RRE site 7837 and T→A at 7855
14. pNL4-3 nef (-) single mutagenized above 13.10 (#3546)-#1272 with NheI to BamHI from mutagenized #3559 with mutation A→G at site 7854 and additional mutation C→A at RRE site 7837

15. pNL4-3 nef (-) single mutagenized below 13.10 (#3547)-#1272 with NheI to BamHI from mutagenized #3559 with mutation A→G at site 7854 and additional mutation T→A at RRE site 7855
16. pNL4-3 nef (-) double mutagenized 13.10 (#3548)-#1272 with NheI to BamHI from mutagenized #3559 with mutation A→G at site 7854 and additional mutations C→A at RRE site 7837 and T→A at 7855
17. R73 (#3549)- infectious proviral clone of HXB2 derivative virus (HXB2 Genbank Accession number KO3455)
18. 13.10 Topoclone (#3559)-pCR4 vector containing NheI to XhoI from Clone 13.10 derived from 104366 resistant variant with mutation A→G at RRE site 7854
19. pSPRRE Peak 7 (#3560)-#1627 site mutagenized to create mutation C→T at RRE site 7836 identified in Peak 7 103833 resistant variant
20. pNL4-3 13.10 (#3561)-#1145 with NheI to BamHI of #3559 creating A→G mutation at RRE site 7854
21. pNL4-3 peak 7 (#3562)-#1145 with NheI to BamHI of #3560 creating C→T mutation at RRE site 7836
22. pNL4-3 rev(-) (#3563)-#1146 with NheI to BamHI of #3559 creating A→G mutation at RRE site 7854 and containing TAT→TAA Rev aa 23 premature stop
23. pNL4-3 rev(-) (#3564)-#1146 with NheI to BamHI of #3560 creating C→T mutation at RRE site 7836 and containing TAT→TAA Rev aa 23 premature stop

24. pCMV (#16)-cytomegalovirus IE94 promoter/enhancer empty vector
25. pCMVRev (#30)-Rev coding region from HIV-1 clone #118 under the control of cytomegalovirus IE94 promoter/enhancer
26. pCMVRev NcoI-XhoI (#843)-#30 with NcoI-XhoI sites created around the Rev coding region
27. pCMVRev NL4-3 (#3612)-#843 with NcoI-XhoI PCR augmented fragment from pUC Rev NL4-3
28. pNL4-3 RRE61 (#3613)-#1145 with NheI to BamHI from #1721 containing G→A RRE sites 7864 and 7945 of RevM10 Clone 61
29. pNL4-3 rev(-) RRE61 (#3614)- #1146 with NheI to BamHI from #1721 containing G→A RRE sites 7864 and 7945 of RevM10 Clone 61 and containing TAT→TAA Rev aa 23 premature stop
30. pCMVGagPol (#3442)- cytomegalovirus IE94 promoter/enhancer driven GagPol RRE containing vector producing noninfectious viral like particles
31. pCMVGagPol 13.10 (#3615)-#3442 with PCR XhoI to BamHI augmented fragment from #3559 containing A→G RRE site 7854
32. pCMVGagPol Peak 7 (#3616)-#3442 with PCR XhoI to BamHI augmented fragment from #3560 containing C→T RRE site 7836
33. pCMVGagPol RRE61 (#3617)-#3442 with PCR XhoI to BamHI augmented fragment from #1721 containing G→A at sites 7864 and 7945

34. R73 SOE 7295 (#3694)-#3549 SOE PCR mutagenized with oligos 379/1802, 1808/1803 then 379, 1808 to create GAT to GCT at R73 nucleotide 7295
35. R73 SOE 6402 (#3695)-#3549 SOE PCR mutagenized with oligos 379/1804, 1808/1805 then 379, 1808 to create GCA to GCC at R73 nucleotide 6402
36. R73 SOE DM (#3696)-#3549 SOE PCR mutagenized with oligos 379/1802, 1808/1803 then 379, 1808 in the first round; oligos 379/1804, 1808/1805 then 379, 1808 in a second round to create GAT to GCT at nt 7295 and GCA to GCC at nt 6402 of R73
37. R73 Rev (-) (#3697)-#3549 SOE PCR mutagenized with 379/1806, 18081/1807 then 379, 1808 to create TAT to TAA in Rev aa 23

Cell Lines

Cell lines listed are referenced from NIH AIDS Research and Reference Reagent Program (www.aidsreagent.org) or ATCC (www.atcc.org). Cells used from in house laboratory stocks.

1. SupT1-lymphoblastic leukemia T cell line. Cells are maintained in RPMI 1640 media (GIBCO) supplemented with 10% Fetal Calf Serum and 50μg/ml Gentamycin Sulfate.
2. CEM-lymphoblastic leukemia T cell line. Cells are maintained in RPMI 1640 media supplemented with 10% Fetal Calf Serum and 50μg/ml Gentamycin Sulfate.

3. 293T-neuronal cells derived from primary human embryonic kidney infected with Adenovirus-5 sheared DNA and Large T antigen. Cells are maintained in IMDM media supplemented with 10% Bovine Calf Serum and 50μg/ml Gentamycin Sulfate.
4. Ghost CXCR4-HOS derived cells with Tat dependent HIV-2 LTR driven GFP reporter. Cells are maintained with IMDM media supplemented with 10% Fetal Calf Serum, 500μg/ml G418, 100μg/ml Hygromycin, 1μg/ml Puromycin, and 50μg/ml Gentamycin Sulfate.
5. CEM G11-lymphoblastic leukemia T cell line with Rev and Tat dependent LTR driven GFP reporter. Cells are maintained in RPMI 1640 media supplemented with 10% Fetal Calf Serum and 50μg/ml Gentamycin Sulfate.
6. 5BD.1- Used by Message Pharmaceuticals, designed in our laboratory. COS derived stable cell line created by transfecting CMT3 cells with pCMVGagPol-RRE, pCMVRev, pCMVEnv. Cells are maintained in IMDM supplemented with 10% Fetal Calf Serum, 0.2mg/ml Hygromycin B, 1.5mg/ml G418, and 0.05mg/ml Gentamycin Sulfate.
7. MT4- Used by Southern Research Institute in MTS toxicity assays. HeLa derived cell line maintained in DHEM and RPMI-1640 (1:1) supplemented with 10% Fetal Calf Serum, penicillin 50U/ml, and streptomycin 0.05ng/ml.

Primary Screening Assay and Dose Response Assay

This assay was performed at Message Pharmaceuticals. 5BD.1 cells used in this assay were previously designed in our laboratory by $CaPO_4$ transfection of pCMV GagPol-RRE, pCMVrev, pCMVenv constructs into CMT3 cells. 5BD.1 cells suspended in medium (IMDM supplemented with 10% Fetal Calf Serum, 0.2mg/ml Hygromycin B, 1.5mg/ml G418, 0.05mg/ml Gentamycin) were added to each well of 384 well plates at a concentration of 4500 cells per 40μl per well. The plates were incubated in the presence of 10μM compound overnight for 16 hours. Wells without compound, and wells without compounds and cells were used as controls (IC100 and IC0). After 16 hours the media was removed and discarded. Fresh 40μl media with 10μM compound was applied and the cells were incubated for an additional 8 hours. 25μl supernatant was collected and used in a p24 ELISA. Compounds that inhibited p24 production by at least 50% were selected as targets (IC50).

5BD.1 cells suspended in medium (IMDM supplemented with 10% Fetal Calf Serum, 0.2mg/ml Hygromycin B, 1.5mg/ml G418, 0.05mg/ml Gentamycin) were added to each well of 96 well plates at a concentration of 20,000 cells per 135μl per well. The plates were incubated in the presence of varying concentrations (30μM, 10μM, 3μM, 1μM, 0.3μM, 0.1μM, or 0 for controls) of the 192 target compounds for 16 hours. The same controls were used as previously described. After 16 hours the media was removed and discarded. 135μl of fresh media with the appropriate concentration of compound was applied and the cells were

incubated for 24 hours. Supernatant was collected and used in a p24 ELISA. Compounds again that inhibited p24 production by at least 50% were selected for further study.

Toxicity of the compounds

Toxicity experiments were performed at Message Pharmaceuticals and Southern Research Institute. 5BD.1 cells were observed microscopically for signs of cellular toxicity at the end of the dose response assays. Toxicity of the compounds was further evaluated by a more stringent assay, the Promega CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay. The assay uses a tetrazolium salt (MTS) and the electron coupling reagent phenazine methosulfate (PMS). MTS is chemically reduced by the cells into formazan that can be measured by absorbance at 492nm. The production of formazan is proportional to the number of living cells. Toxicity is then derived from this value.

HeLa derived MT-4 cells were suspended in media (DHEM, RPMI-1640 supplemented with 10% Fetal Calf Serum, penicillin 50U/ml, streptomycin 0.05ng/ml) and transferred to each well of 96 well plates at a concentration of 200,000 cells per 100μl per well. The plates were incubated for 5 days with the same compound concentrations used in the dose response assays. On the last day the test reagents MTS and PMA were added per manufacturers instructions (Promega) at a ratio of 1:5 (detection reagent: well content). Absorbance was measured at 492nm to

determine the % living cells and to derive the Toxicity of the compounds (TC50).

Dual Luciferase Rev Assay

The Dual Luciferase Rev Assay was performed at Southern Research Institute. The Rev Assay utilizes a stable HeLa derived cell line that contains an integrated bicistronic expression construct of both HIV-1 IIIB *rev* gene and Firefly Luciferase gene under the control of a single Tet-Off promoter, and a HIV-1 SF2 Rev-dependent Renilla Luciferase reporter construct. Cells are assayed in DHEM media supplemented with 10% Fetal Calf Serum, penicillin 50U/ml, and streptomycin 0.05ng/ml. Cells containing the Rev-dependent Renilla Luciferase and Rev-independent Firefly Luciferase reporters were added to wells of 96 well plates in the concentration of 20,000 cells per 200μl per well. The plates were incubated for 24hours with decreasing ½ log concentrations of compound. The media was removed, the cells lysed, and then the luciferases measured per manufacturers instructions (Promega). The therapeutic index of this assay corresponds to the inhibition of Renilla versus Firefly luciferase compared to a control (IC50 Firefly/IC50 Renilla).

Specificity for RRE using CTE Construct Control

Fei Fei Chen performed this assay at our laboratory. 5μg of pCMVGagPol constructs containing either RRE or MPMV CTE and 1ng

pCMV Rev were transfected into 293T cells using a standard $CaPO_4$ protocol. Various concentrations of the compounds (0, 1.875μM, 3.75μM, 7.5μM, 15μM) were tested by suspension in the transfection culture media (IMDM supplemented with 10% Fetal calf serum, 50μg/ml Gentamycin). Supernatants were harvested 72 hours post transfection and evaluated for p24 by ELISA. The CTE constructs are Rev-independent, compared to the RRE constructs that require Rev for expression. The assay shows specificity of the inhibitory compounds for the Rev-RRE pathway.

Inhibition of HIV-1 release from induced U1 cells

This assay was performed at Southern Research Institute. U1 cells (NIH AIDS Research and Reference Reagent Program) were suspended in media (RPMI supplemented with 10% Fetal Calf Serum, 100U/ml penicillin, 100μg/ml streptomycin) and added to wells of 96 well plates at concentration of 50,000 cells per ml. Cells were induced with 5ng/ml TNF-alpha added to the well contents. Compounds were also added at various concentrations (103833: 0, 0.12μM, 0.4μM, 1.2μM, 3.8μM, 11.9μM; 104366: 0, 0.25μM, 0.8μM, 2.5μM, 7.9μM, 25.1μM). Cultures were incubated for 3 days and the supernatants harvested for reverse transcriptase analysis. Toxicity was determined by MTS dye reduction of the cells as previously described.

In-Vitro Gel Shift Assay

Sharmila Raman performed this assay at our laboratory. RRE plasmids were linearized using XhoI or MluI, and then purified using a standard phenol/chloroform extraction and precipitation. We performed in vitro transcription of the RRE RNA probe using P32-UTP. The probe was made under the following conditions: 2μl 10x T7 buffer containing 0.1mM DTT, 0.5μl RNAsin, 4μl 2.5mM rNTPs-UTP, 2μl 50μM UTP, 1μl linearized RRE template 1μg/μl, 5μl 32P-UTP (3000Ci/mmol), 2μl T7 RNA polymerase, and 4μl DEPC treated dH_20 incubated at 37°C for 2 hours. The reaction was treated with RQ1 DNAse at 37° for 30 minutes to remove the DNA template. The probe was run on a 4% Urea Acrylamide gel. The RNA band in the gel was identified on exposed X-ray film, and then cut out of the appropriate region. The gel slice was suspended in TE and shaken at 4°C overnight. The RNA was extracted using phenol pH 6.6/chloroform extractions, and then precipitated with 3M pH 5.2 Sodium Acetate and 2 Volumes 100% Ethanol overnight at –80°C. The RNA precipitate was washed with 70% ethanol and allowed to air dry. The RNA was suspended in TE and purified again using a BD Biochem Chromaspin 30 column per manufacturers instructions. The RNA was folded by suspension in a Renaturation buffer (500mM NaCl, pH 7.8 100mM HEPES/KOH, 20mM $MgCl_2$), incubation at 80° for 3 minutes, then cooling to 4°. 0.5ng of this probe was incubated with 38ng of Rev protein in Rev-RNA binding buffer (200mM KCl, pH 7.8 20mM HEPES/KOH, 4mM $MgCl_2$, 1mM EDTA, 2mM DTT, 20% glycerol) on ice for 10 minutes.

Compounds were added to the reaction at various concentrations to test for inhibition of Rev-RRE association in this in vitro system. The reaction was run on 4% Urea Acrylamide gel in 1x TBE. The gel was dried and then exposed to a phosphoimager screen overnight. The assay was quantitated using a phosphoimager and the following formula:

Relative shift=(c-b)/(c-b+p)/S

c=Rev-RRE complex cpm, p=uncomplexed probe, b=radioactive drag, S=% shift at full complex formation

Inhibition of HIV-1 Replication in PBMCs

Southern Research Institute performed this assay. PHA-P stimulated PBMCs from two normal blood donors were pooled, diluted in fresh medium to a concentration of 1 million cells per ml, and plated in 96 well plates at 50μl per well per SRI protocol. NL4-3 and BR/93/021 (R5 tropic Subtype B HIV-1 patient isolate) were used to infect the cells at MOI of 0.1. The cultures were incubated for 7 days, and then supernatant was analyzed for reverse transcriptase activity. Cytotoxicity was measured by MTS toxicity assay as previously described.

Generation of Viral Stock

Proviral Transfection

Viral stocks were generated from $CaPO_4$ Transfection of 293T cells with plasmids containing the proviral DNA. The day before transfection confluent plates of 293T cells were spit into 25cm² flasks by trypsin

digestion to yield a concentration of $1x10^6$ cells in 5ml IMDM with10% Fetal Calf Serum and 50μg/ml Gentamycin Sulfate. For transfection, 5μg of proviral DNA was mixed with 100μl 2.5M pH 7.2 Calcium Chloride in 10mM HEPES and dH_20 up to a total of 1ml. This solution was added dropwise to 1ml 2x HEBS (HEPES Buffered Saline) pH 7.2 while slowly bubbling for gentle mixing. The precipitation was done at room temperature for 30 minutes. The DNA precipitate was added to the media of the 293T cells dropwise, and then incubated 4 hours at 37°C, 4.5% CO_2, and 85% humidity. After incubation the media was removed and replaced with 5ml IMDM supplemented with 10% Bovine Calf Serum and 50μg/ml Gentamycin Sulfate. The transfected cells were incubated as described in BL3 conditions for 3 days for viral production. Supernatant collected on day 3 was used for p24 analysis and infection of T cell lines.

T cell Infection

Viral stocks were produced from infection of SupT1 or CEM T cell lines with virus derived from either transfection or culture supernatants. Cells (CEM G11 or SupT1) were split and placed in 6 well plates at the concentration of 5 million cells per well in 2ml RPMI media supplemented with 10% Fetal Calf Serum and 50μg/ml Gentamycin Sulfate. 200ng of Hexadimethrine Bromide (polybrene®) was added to each well and the plate was incubated 30 minutes at 37°C. In BL-3 conditions 50-125ng p24 equivalents of virus was added to the corresponding well and the plate

was incubated at 37°C, 4.5% CO_2, and 85% humidity for 4 hours. Upon completion of the incubation, the 2ml well contents were transferred to corresponding 15ml conical tubes then centrifuged at 3000 rpm for 5 minutes. For each tube the supernatant with noninfectious virus was discarded, the infected cells suspended in 5ml RPMI media supplemented with 10% Fetal Calf Serum and 50μg/ml Gentamycin Sulfate, and the resultant cultures placed in 25cm² flasks. The viral cultures were incubated at 37°C, 4.5% CO_2, and 85% dH_2O in BL3. Every 3 days the cultures were split removing 3ml of each culture, then adding back 3ml of fresh media to each culture flask. The removed portions of the cultures were separated by centrifugation for samples of supernatant (containing viral particles) and infected cells. Samples were stored at –80°C. Viral growth was determined by p24 ELISA.

Measurement of p24 by ELISA

The p24 levels of viral culture or transfection supernatants were measured by an in house p24 ELISA. A 96 well plate was incubated overnight at 37°C containing 100μl of the primary antibody (monoclonal IgG p24) in each well. The next day the plate was washed with 1x PBS. 250μl of blocking buffer (PBS with 5% BSA) was added to each well and the plate incubated at 37°C for 1 hour. The plate was then washed with 1x PBS with 0.5% Tween-20. 10μl of lysis buffer (1x PBS with 10% Triton X-100 and 0.05% Trypan Blue) was added to each well except for the

blanks. In BL3 conditions 100μl of standard or sample dilutions were added to appropriate wells. The plate was then incubated at 37°C for at least 2 hours. The plate was washed with 1x PBS with 0.5% Tween-20. 100μl of biotinylated secondary antibody diluted 1:800 in assay buffer (PBS with 10% Bovine Calf Serum and 0.5% Triton X-100) was added to all wells except the blanks. The plate was incubated at 37°C for 1 hour. The plate was washed with 1x PBS with 0.5% Tween. 100μl of peroxidase-streptavidin diluted 1:4000 in assay buffer was added to all the wells except the blanks. The plate was incubated at 37°C for 30 minutes. The plate was washed with 1x PBS with 0.5% Tween. 100μl of peroxidase substrate (OPD) was added to every well. The plate was incubated at room temperature protected from light for 30 minutes. 50μl of 2M H2SO4 stop solution was added to each well and the optical density was measured at 492nm. The p24 values (ng/ml) were calculated by the comparison of the measured intensity values to the standard curve factoring in the dilution of the samples.

Viral Stock Titration

In addition to measuring p24 content we utilized CEM G11 reporter cells to do end point titration of the viral stock. CEM G11 cells effectively measure viral infection because the reporter requires the viral proteins Tat to augment transcription of the GFP reporter, and Rev to transport the reporter message for translation. The cells were split and placed in 24

well plates at a concentration of $5x10^5$ cells per well suspended in 0.2ml RPMI media supplemented with 10% Fetal Calf Serum and 50μg/ml Gentamycin Sulfate. 100ng of Hexadimethrine Bromide (polybrene®) was added to each well and the plate was incubated 30 minutes at 37°C. In BL-3 conditions 100μl serial (10 fold) dilutions in quadruplicate of virus was added to the corresponding well and the plate was incubated at 37°C, 4.5% CO_2, and 85% humidity for 2 hours. 1.7ml additional media was added to each well and the plate was incubated for an additional 4 days. The well contents were removed into 15ml conical tubes and the contents centrifuged at 3000rpm for 5 minutes. The supernatant was sampled for p24 ELISA. The cells were washed with PBS then fixed with 2% paraformaldehyde in PBS for 24 hours at 4°C. The cells were analyzed compared to a CEM G11 negative control for the expression of GFP. The Reed-Muench formula was used to determine the TCID50 of the viral stock based on the dilution at which 50% of the wells were positive for viral replication.

Cellular Toxicity of Rev-RRE Inhibitor Compounds

Prior to beginning the long-term variant selection experiment, the 104366 compound was tested for generalized toxicity in the T cell lines. CEM and Sup T1 cell lines were cultured in the presence of 104366 for a total of 2 weeks at concentrations of 0, 1.77, 5.3,15.9μM in duplicate. Cell viability counts were done every 3 days to access toxicity using a

hemocytometer and Trypan blue viability stain of cell samples. Cell death was noted only at the 15.9μM concentration as was expected from previous toxicity experiments performed by our collaborators at Southern Research Institute.

Long Term Selection of Resistant Variants

Cells (CEM G11 or SupT1) were split and placed in 6 well plates at the concentration of 5 million cells per well suspended in 2ml RPMI media supplemented with 10% Fetal Calf Serum and 50μg/ml Gentamycin Sulfate. 200ng of Hexadimethrine Bromide (polybrene®) was added to each well and the plate was incubated 30 minutes at 37°C. In BL-3 conditions 50-125ng p24 equivalents of NL4-3 nef (-) virus was added to the corresponding well and the plate was incubated at 37°C, 4.5% CO_2, and 85% humidity for 4 hours. Upon completion of the incubation, the 2ml well contents were transferred to corresponding 15ml conical tubes then centrifuged at 3000 rpm for 5 minutes. For each tube the supernatant with noninfectious virus was discarded, the infected cells suspended in 5ml RPMI, 10% Fetal Calf Serum, and 50μg/ml Gentamycin Sulfate media or the same media with diluted Rev-RRE inhibitor compound, and the resultant cultures placed in 25cm² flasks. The viral cultures were incubated at 37°C, 4.5% CO_2, and 85% dH_2O. Every 3 days the cultures were split removing 3ml of each culture, then adding back 3ml of media or media with the appropriate concentration of compound to each culture

flask. 104366 concentrations included 0.8μM (IC50) and 8μM. 103833 concentrations included 1μM (IC50) and 10μM. The removed portions of the cultures were separated by centrifugation for samples of supernatant (containing viral particles) and infected cells. Samples were stored at –80 °C. Viral growth was measured by p24 ELISA as previously described. Cultures were also monitored for cytopathic effects (cell size, giant cell formation, cell lysis) by light microscopy of the culture flasks in BL3 conditions.

Viral Testing for Resistance

When an increase in the p24 level was observed for the long term selection cultures exposed to compound (indicating viral growth) the viral supernatant was used to infect T cell lines in comparison to the original NL4-3 nef (-) virus that was sensitive to the compounds. Infections were performed as previously described either in the absence of compound, or at 104366 concentrations of 0.8μM and 8μM or 103833 concentrations of 1μM and 10μM. When the new viral variant was able to replicate in the presence of compound at the concentrations tested, the resistant variant was isolated for further study. Supernatant and cell samples were collected for these cultures and stored at –80°C.

Variant Testing for Cross Resistance

Viral supernatant from resistant cultures were used to infect T cells in the presence of the other inhibitor compound. Cultures were split every

3 days and supernatant was tested by p24 ELISA. Growth curves were produced with measured p24 values over time to illustrate cross resistance. Cultures were compared to wild type NL4-3 nef (-) cultures grown in tandem that were sensitive to the compounds.

Variant Testing for Reversion

Viral supernatant from resistant cultures was used to infect T cells in the absence of inhibitor compound. Cultures were split every 3 days and supernatant was tested by p24 ELISA. Growth curves were produced with measured p24 values over time. Viral supernatant from the peak of these cultures was used to infect T cells in the presence of inhibitor compound. Cultures were split every 3 days and supernatant was tested by p24 ELISA. Growth curves were produced with measured p24 values over time and evaluated for loss of resistance. Cultures were compared to wild type NL4-3 nef (-) cultures grown in tandem that were sensitive to the compounds. Regression cultures were done in this manner for 10 passages.

Viral RNA Isolation

1ml of viral culture supernatant was added to a BECKMAN tube and ultra centrifuged for 1 hour at 30,000 rpm at 4°C. The supernatant was carefully decanted by pipette, and then the pellet was suspended in 20µl RQ1 DNAse 1x Buffer (NEB). 5µl RQ1 DNAse (1unit/µl) was added

incubating at 37°C for 30 minutes. 75µl TE buffer pH 7.4, 10µl yeast t-RNA (1µg/µl), 6µl 10% SDS, and 3µl proteinase K (10mg/ml) were added incubating at 42°C for 30 minutes. 100µl phenol pH 6.6 and 100µl chloroform were added, the tube vortexed then centrifuged at 13000 rpm for 5 minutes. The top layer was removed and added to a clean tube. The phenol-chloroform extraction was repeated. The top layer was removed and added to a clean tube. 13µl 3M sodium acetate and 325µl 100% ethanol were added incubating at –80°C for 1 hour. The tube was centrifuged at 13000 rpm for 15 minutes at 4°C. The supernatant was decanted and pellet washed with 75% ethanol twice. The RNA pellet was allowed to air dry then was suspended in 50µl 10mM Tris pH 7.4 and stored at –80°C.

RT PCR Viral RNA

Viral RNA was amplified into cDNA by the following RT-PCR procedure. Reaction mix 1x was prepared as listed: 10x $MgCl_2$ PCR Buffer 5µl, DEPC treated dH_2O 35.5µl, dNTP (10mM) 0.4µl, Taq Pol 0.125µl, AMV RT 0.2µl, RNAsin 0.25µl, $MgCl_2$ 1.5µl (43µl total each reaction tube). Primers 1µl each (forward and reverse) were added to each reaction tube. RNA template 5µl was added to the reaction tubes. Control tubes missing RNA template or containing RNA template but missing AMV RT were also run to analyze for reaction contamination. The reactions were run at the following cycling conditions: 42°C for 1 hour for reverse transcriptase,

95°C for 3 minutes to inactivate the AMV RT, (95°C 1 minute, 58°C 1 minute, 72°C 2 minutes) 30 cycles of strand separation, annealing, extension, 72°C for 10 minutes final extension, and 4°C for DNA storage.

Primers used for RT-PCR reaction:

For amplification of the RRE:

Forward/Upper

396-5’ TAA ACA TGT GGC AGG AAG TAG G 3’ (22mer)

Reverse/Lower

23-5’ GTT CAC TAA TCG AAT AAT GGA TCT G 3’ (25mer)

245-5’ GGC CTG TCG GGT CCC CTC GGG 3’ (21mer)

For amplification of Rev exon 1 with SalI to NheI sites:

Forward/Upper

299-nt 5562-5579-5’ GCG GGA TCC GAA CAA GCC CAA GAA GAC 3’ (27mer)

Reverse/Lower

234-nt 6334-6354-5’ GCG AAT TCA CAC AGG TAC CCC ATA ATA 3’ (27mer)

128-5’ TTA CAG TAG AAA AAT TCC CC 3’ (20mer)

For amplification of Rev exon 2:

Forward/Upper

366-nt 8091-8115-5’ TGA CCT GGA TGG AGT GGG ACA GAG A 3’ (25mer)

Reverse/Lower

190-nt 8814-8838-5’ GCT GCT GTG TTG CTA CTT GTG ATT G 3’ (25mer)

For amplification of RRE and Rev exon 2 with NheI and XhoI sites:

Forward/Upper

1558-nt 7105-7125-5’ AAG ACC CAA CAA CAA TAC AAG 3’ (21mer)

Reverse/Lower

1560-nt 9005-9026-5’ AGT CAT TGG TCT TAA AGG TAC C 3’ (22mer)

Nested PCR of cDNA

NESTED PCR of RT-PCR cDNA product was performed to further amplify fragments of interest.

Reaction mix 1x was prepared as listed: 10x Buffer 10μl, dH_2O 76.95μl, dNTP 0.8μl, Taq Pol 0.25μl, $MgCl_2$ 3μl (91μl total each reaction tube). Primers 2μl each (forward and reverse) were added to each reaction tube. 5μl of the cDNA was added to each appropriate reaction tube. Reactions were performed under the following cycling conditions: 95°C for 2 minutes, (95°C 1 minute, 45°C 1 minute, 72°C 2 minutes) 30 cycles of strand separation, annealing, extension, 72°C for 10 minutes for final extension, and 4°C for cDNA storage.

Primers used for Nested PCR reaction:

Primers used to amplify the RRE:

Forward/Upper

1438-nt 7712-7733-5' AAG GCA AAG AGA AGA GTG GTG C 3' (22mer)

Reverse/Lower

1439-nt 8018-8039-5' GCA CAG CAG TGG TGC AAA TGA G 3' (22mer)

Primers used to amplify Rev exon 1 with SalI to NheI sites:

379-5' CAA TGA ATG GAC ACT AGA GC 3' (20mer)

479-5' TCC CCT CCT GAG GAT TGC 3' (18mer)

Primers used to amplify the RRE and Rev exon 2 with NheI and XhoI sites:

Forward/Upper

1557-nt 7199-7221-5' GCA CAT TGT AAC ATT AGT AGA GC 3' (23mer)

Reverse/Lower

1559-nt 8911-8932-5' GCT GTA TTG CTA CTT GTG ATT G 3' (22mer)

DNA Sequencing

DNA was evaluated for mutations, using the UVA DNA Sequencing center, by dye terminator chemistry with Taq polymerase and primers overlapping the fragments of interest. Sequencing was performed on

purified nested PCR product and molecular clone DNA produced as described.

Molecular Cloning

To further study the fragments of interest amplified from the viral mutants, molecular cloning was performed using Invitrogen's TA TOPO Cloning kit for sequencing per manufacturer instructions. This allowed direct insertion of Taq polymerase amplified PCR products into the plasmid vector for sequencing. The pCR4 TOPO plasmid contains single T overhangs and Topoisomerase covalently bound to the vector. Taq polymerase has a nontemplate-dependent terminal transferase activity that adds a single A to the 3' ends of PCR products. Topoisomerase I from Vaccinia virus binds to duplex DNA and cleaves the phosphodiester backbone after 5'CCCTT in one strand. The energy from the broken phosphodiester backbone is conserved by formation of a covalent bond between the 3' phosphate of the cleaved strand and between a tyrosyl residue (Tyr-274) of topoisomerase I. The phospho-tyrosyl bond between the DNA and enzyme can subsequently be attacked by the 5' hydroxyl of the original cleaved strand, reversing the reaction and releasing topoisomerase. DH5- a T1 One Shot Chemically Competent cells (F-psi80lacZdeltaM15 deltalacX74 hsdR(rk-, mk+) deltarecA1398 endA1 tonA) were transformed with the pCR4 vector containing fragments from the nested PCR product. The pCR4 TOPO vector allows direct positive

selection by disruption of the lethal ccdB gene by insert of PCR product. Cells that contain non-recombinant vectors are killed upon plating. Also the vector contains genes for antibiotic resistance for plating on antibiotic selection media (LB agar plates with 50μg/ml kanamycin or 100μg/ml ampicillin).

TOPO Cloning Reaction were performed as follows: Purified PCR Product 4μl, Salt Solution 1μl, and TOPO Vector 1μl (total 6μl) were added to tubes then incubated at RT for 20 minutes. DH5-a cells were transformed by adding 6μl TOPO Cloning reaction per thawed vial of cells, and then incubating on ice for 30 minutes. The cells were then heat shocked for 30 seconds at 42°C then transferred to ice. 250μl SOC media was added to the cells and they were incubated at 37°C shaking horizontally at 210 rpm for 1 hour. 200μl from each transformation reaction was spread on LB agar plates then incubated at 37°C overnight. 10 colonies were picked for analysis and the rest were stored in LB with 15% glycerol at –80°C.

Plamid Miniprep

A clonal colony was cultured overnight in 5ml LB broth with 50μg/ml kanamycin or 100μg/ml ampicillin. 0.5ml of culture was mixed into 0.5ml LB with 30% glycerol and stored at –80°C. The rest of the culture was centrifuged at 3000 rpm for 15 minutes. The media was decanted and the bacterial pellet prepped with Qiagen miniprep kit per manufacturers

instructions. The plasmid was evaluated for size and concentration by 1 % agarose gel electrophoresis compared to lambda Hind III digest and 1kb Invitrogen ladders. DNA sequencing as previously described was used to evaluate fragments of interest within the clones.

Plasmid Maxiprep

A clonal colony was cultured overnight (37° DH5-a cells, 30° Stbl 2 cells) in 5ml LB broth with 50μg/ml kanamycin or 100μg/ml ampicillin. The 5ml culture was used to seed 250ml LB broth with 50μg/ml kanamycin or 100μg/ml ampicillin for overnight growth. The culture was centrifuged at 3000 rpm for 15 minutes. The media was decanted and the bacterial pellet prepped with Qiagen maxiprep kit per manufacturers instructions. The plasmid was evaluated for size and concentration by 1 % agarose gel electrophoresis compared to lambda Hind III digest and 1kb Invitrogen ladders. DNA concentration was also analyzed by a spectrophotometer at 260nm. DNA sequencing was used to evaluate fragments of interest within the clones.

Directional cloning of mutant fragments into wild type proviral backbone

Either SalI/NheI or NheI/XhoI restriction enzymes were used to digest the clones (containing mutant fragment of interest) and the wild type provirus. The restriction digests were separated using 1% agarose

gel electrophoresis in 1x TAE. Bands containing the mutant fragment or the wild type backbone were excised from the gel and the DNA was isolated by Qiagen gel extraction kit per manufacturer's instructions. The mutant DNA SalI/NheI or NheI/XhoI fragments were ligated to the wild type provirus using T4 DNA ligase (NEB). The ligation product was analyzed on 1% agarose gel in 1xTAE. The ligated DNA was excised from the gel and extracted by Qiagen gel extraction kit per manufacturers instructions. 15μl of the proviral DNA was used to transform Invitrogen Stbl 2 competent cells. Transformation of Stbl 2 cells was performed in a similar manner to DH5-a cells except for the following differences: recovery in SOC broth done at 30°C shaking horizontally at 200rpm for 1 ½ hours; because the proviral backbone only contains the ampicillin resistance gene, LB with 100μg/ml ampicillin was used to grow bacteria at 30°C. The Stbl 2 cells containing provirus were grown first in 5ml, then 250ml LB with 100μg/ml ampicillin. Plasmid maxiprep was performed as described. Restriction digest, 1% agarose gel electrophoresis, spectrophotometry, and DNA sequencing were used to analyze the proviral DNA. Proviral DNA containing mutations of interest were then used in $CaPO_4$ Transfection of 293T cells as previously described. Resultant viruses were used for infection of T cells.

Cloning of 103833 resistance mutation

Viral RNA from the 103833 resistant variant was isolated in the same manner described. DNA sequencing of the nested PCR products identified a single point mutation in the stem IIC of the RRE. This mutation was created by site-directed mutagenesis of the NL4-3 RRE in plasmid #1627 using the following oligos:

1621 5' CAATGACGCTGACGGTACAGGTCAGACAATTATTG 3'

1625 5" CAATAATTGTCTGACCTGTACCGTCAGCGTCATTG 3'

The mutagenesis was done using Stratagene Quikchange XL Site-Directed Mutagenesis kit per manufacturers instructions. The mutagenized plasmid and wild type provirus were sequentially digested with restriction enzymes NheI and BamHI. The restriction digests were separated using 1% agarose gel electrophoresis in 1x TAE. Bands containing the mutant fragment or the wild type backbone were excised from the gel, and the DNA was isolated by Qiagen gel extraction kit per manufacturers instructions. The mutant DNA NheI/BamHI fragment was ligated to the wild type provirus using T4 DNA ligase (NEB). The ligation product was analyzed on 1% agarose gel in 1xTAE. The ligated DNA was excised from the gel and extracted by Qiagen gel extraction kit per manufacturers instructions. 15μl of the proviral DNA was used to transform Invitrogen Stbl 2 competent cells. Transformation of Stbl 2 cells was performed in a similar manner to DH5-a cells except for the following differences: recovery in SOC broth done at 30°C shaking horizontally at 200rpm for 1 ½ hours; LB with 100μg/ml ampicillin was used to grow

bacteria at 30°C (provirus only contains ampicillin resistance gene). The Stbl 2 cells containing provirus were grown first in 5ml, then 250ml LB with 100μg/ml ampicillin. Plasmid maxiprep was performed as described. Restriction digest, 1% agarose gel electrophoresis, spectrophotometry, and DNA sequencing were used to analyze the proviral DNA. Proviral DNA containing the mutation of interest was then used in $CaPO_4$ Transfection of 293T cells as previously described. Resultant virus was used for infection of T cells.

Viral growth to confirm mutations that confer resistance to 103833 and 104366

Mutant virus was used to infect SupT1 T cells as previously described without the presence of compound, with 1 or 10μM 103833, or with 0.8 or 8μM 104366. The cultures were split every 3 days and supernatant was removed for p24 ELISA. Growth curves showing p24 values over time were created demonstrating viral sensitivity or resistance to the compounds. Virus containing mutations identified during selection with one compound were grown in the presence of the other compound to test for cross resistance. Mutant viruses were cultured in tandem with wild type NL4-3 nef (-) viral cultures still sensitive to the compounds.

Site Directed Mutagenesis of Stem Loop IIC of Mutant Viruses

Resistance mutations were identified in the RRE stem loop IIC that also changed the amino acid sequence of gp41. Site directed mutagenesis was performed to disrupt the stem loop while maintaining the amino acid sequence conferred by the resistance mutations. This experiment separated the two possible factors conferring the resistance phenotype in the variants. Single point mutations were made in the third amino acid in the codons containing the resistance mutations. Stratagene Quickchange Site-Directed mutagenesis kit was used to introduce the desired mutations in the resistant mutant RREs. The initial PCR reaction consisted of the following: 10X reaction Buffer 5μl, 50ng plasmid (#3559 or #3560), 125ng Primer 1, 125ng Primer 2 (complementary sequence), dNTP (10mM) 1μl, dH_20 up to 50μl, PfuTurbo DNA polymerase 1μl. The reaction was conducted under the following cycling parameters: 95°C for 30 seconds, (95°C 30 seconds, 55°C 1 minute, 68°C 6 minutes) x 16 cycles, 68°C for 10 minutes, 4°C. 1μl DpnI per reaction tube was added and the backbone DNA digested at 37°C for 1 hour. XL1 competent cells were transformed by adding 4μl reaction to 50μl cells and incubating on ice for 30 minutes. The cells were then heat shocked for 45 seconds at 42°C then returned to ice. 500μl NZY+ broth was added to the tube incubating shaking horizontally at 250rpm at 37°C. 250μl was spread on LB agar plates with 100μg/ml ampicillin and incubated overnight at 37°C. Individual clones were expanded in LB broth with 100μg/ml ampicillin overnight then miniprepped as previously described. The mutagenized

RRE sequences were confirmed by DNA sequencing. The clones identified with the desired mutations were digested with the restriction enzymes NheI/XhoI or BamHI along with pNL4-3 nef (-) (#1272). RRE fragments and the wild type backbone were separated and excised from 1% agarose gel in 1xTAE. Fragment DNA was purified from the gel using Qiagen gel extraction kit using manufacturers instructions. The mutagenized RREs were ligated into the wild type backbone using T4 DNA ligase. Ligation reactions were analyzed on 1% agarose gel and then extracted as described. 15μl of resultant proviral DNA containing the RRE mutations was used to transform Invitrogen Stbl 2 competent cells. Transformation of Stbl 2 cells was performed in a similar manner to DH5-a cells except for the following differences: recovery in SOC broth done at 30°C shaking horizontally at 200rpm for 1 ½ hours; LB with 100μg/ml ampicillin was used to grow bacteria at 30°C. The Stbl 2 cells containing provirus were grown first in 5ml, then 250ml LB with 100μg/ml ampicillin. Plasmid maxiprep was performed as described. Restriction digest, 1% agarose gel electrophoresis, spectrophotometry, and DNA sequencing were used to analyze the proviral DNA. Proviral DNA containing mutations of interest was then used in $CaPO_4$ Transfection of 293T cells as previously described. Resultant viruses were used for infection of T cells.

Complementary primers used for site-directed mutagenesis

1621/1625:CAATGACGCTGACGGTACAGGTCAGACAATTATTG/CAATA
ATTGTCTGACCTGTACCGTCAGCGTCATTG
1622/1626:CAATGACGCTGACGGTACAGGCAAGACAATTATTG/CAATA
ATTGTCTTGCCTGTACCGTCAGCGTCATTG
1623/1627:AATTATTGTCTGGAATAGTGCAGCAGCAGCAGAAC/GTTCT
GCTGCTGCTCTATTCCAGACAATAATT
1624/1628:GACGGTACAGGCAAGACAATTATTGTCTGGAATAGTGCAG
CAGCAG/TGCTGCTGCACTATTCCAGACAATAATTGTCTTGCCTGTAC
CGTC

Testing of stem loop IIC mutagenized viruses

Virus with mutagenized stem loop IIC was used to infect SupT1 cells as previously described in the absence of compound and in the presence of 103833 or 104366. Cultures were split every 3 days and samples measured by p24 ELISA. Viral growth was compared to wild type virus inhibited by the compounds and original resistant mutants without the additional stem loop IIC changes.

Western Blot Analysis of U1 cells from SRI

U1 cells from SRI induction experiments were used for western blot analysis of p24 and nef protein levels in the cells. Cell samples included cells that were not induced or were induced with TNF-alpha, and cells that were not exposed to Rev-RRE compound or exposed to either 5μM

104366 or 10μM 103833. Cell pellets were suspended in 200μl dH_20 and 200μl 2x Sample Buffer then mixed by vortexing and boiled for 15 minutes. The samples were run on 14% Polyacrylamide Gel in 1x SDS buffer (10X SDS: Trizma 30.3g, Glycine 148.6g, SDS 10.0g, 1 L dH_2O). Western blot was performed as follows: Millipore FL membrane 3x3.5" activated with methanol then soaked in 1x TB for >15 minutes (10X Transfer Buffer-MeOH: Trizma 33.3g, Glycine 144g, 1 L H20; Final 1x Transfer buffer: 200ml 10X Transfer Buffer-MeOH, 200ml Methanol, 1.6L H20). Finished gel soaked in 1x Transfer Buffer >1 minute. Transfer tank filled to start level with 1x Transfer Buffer. Six 4x4" Whatman paper soaked in 1x Transfer Buffer. Transfer sandwich made in following order: cassette, mesh pad, three Whatman paper, gel, membrane with identifying pencil mark upper right corner (lay mark upper L corner on gel face down), three Whatman paper (bubbles rolled out with pipette), mesh, and cassette. Sandwich closed evenly and put in tank with membrane side to positive electrode allowing protein to move from gel to membrane. Transfer run at 85V for 60min. Membrane removed and blocked with 5% dry milk in PBS with 0.01% Tween 20 for 30 minutes at room temperature. The membrane was rinsed with PBS, and then washed with PBS 0.01% Tween 20 for 1 hour replacing the wash solution every 15 minutes. The primary antibody in PBS with 2% BSA and sodium azide was then applied to the membrane overnight at 4°C. Primary antibodies included laboratory p24 and Nef monoclonal antibody IgG stocks. The membrane was rinsed

with PBS, and then washed with PBS 0.01% Tween 20 for 1 hour. Polyclonal IgG beta tubulin antibody (Abcam) in PBS with 2% BSA and sodium azide was applied to the membrane for 1 hour at room temperature. The membrane was rinsed with PBS then washed with PBS 0.01% Tween 20 for 1 hour. The secondary antibody diluted in 5% dry milk in PBS with 0.01% Tween 20 was applied to the membrane for 1 hour at room temperature. Secondary antibody included anti-mouse (p24 or nef) or rabbit (beta tubulin) infrared-tagged monoclonal antibodies (IRDye 800 anti-mouse IgG Rockland Immunochemicals, Alexa Fluor 680 anti-rabbit IgG Invitrogen). The membrane was rinsed with PBS then washed with PBS 0.01% Tween 20 for 1 hour. A final rinse with PBS was performed before the Odyssey Infrared Scanner was used to analyze the blot. Nef and p24 protein levels were normalized to cellular beta tubulin levels to account for differences in sample loading. The final p24 and nef values were compared to determine Rev-RRE selective inhibition by the compounds.

Insertion of Mutant RRE into pNL4-3 and pNL4-3 Rev (-)

#3559 and #3560 were sequentially digested with NheI and BamHI along with pNL4-3 and pNL4-3 Rev (-) (#1145 and #1146). The restriction digests were separated using 1% agarose gel electrophoresis in 1x TAE. Bands containing the mutant fragment or the wild type backbone were excised from the gel and the DNA was isolated by Qiagen gel extraction

kit per manufacturers instructions. The mutant DNA NheI/BamHI fragments were ligated to the backbone provirus using T4 DNA ligase (NEB). The ligation product was analyzed on 1% agarose gel in 1xTAE. The ligated DNA was excised from the gel and extracted by Qiagen gel extraction kit per manufacturers instructions. 15μl of the proviral DNA was used to transform Invitrogen Stbl 2 competent cells. Transformation of Stbl 2 cells was performed in a similar manner to DH5-a cells except for the following differences: recovery in SOC broth done at 30°C shaking horizontally at 200rpm for 1 ½ hours; LB with 100μg/ml ampicillin was used to grow bacteria at 30°C. The Stbl 2 cells containing provirus were grown first in 5ml, then 250ml LB with 100μg/ml ampicillin. Plasmid maxiprep was performed as described. Restriction digest, 1% agarose gel electrophoresis, spectrophotometry, and DNA sequencing were used to analyze the proviral DNA. Proviral DNA containing mutations of interest was then used in $CaPO_4$ Transfection of 293T cells.

Transfection of NL4-3 with wild type or mutant RREs in presence of compounds

293T cells were transfected by previously described $CaPO_4$ method with 5μg #1145 compared to 5μg provirus containing mutated RREs (#3561, #3562) in the absence or presence of compound. Supernatant from day three was used to compare p24 levels in the various transfection cultures.

Western Blot Analysis of 293T cells from NL4-3 Transfections

Cells from day three of the transfection cultures were manually removed from the flasks. Cells were washed with PBS and concentrated into a pellet using centrifugation at 3000rpm for 5 minutes. The cell pellet was suspended in 200μl dH_20 and 200μl 2x Sample Buffer and boiled for 15 minutes. The samples were run on 14% Polyacrylamide Gel in 1x SDS buffer (10X SDS: Trizma 30.3g, Glycine 148.6g, SDS 10.0g, 1 L dH_2O). Western blot was performed as follows: Millipore FL membrane 3x3.5" activated with methanol then soaked in 1x TB for >15 minutes (10X Transfer Buffer-MeOH: Trizma 33.3g, Glycine 144g, 1 L H20; Final 1x Transfer buffer: 200ml 10X Transfer Buffer-MeOH, 200ml Methanol, 1.6L H20). Finished gel soaked in 1x Transfer Buffer >1 minute. Transfer tank filled to start level with 1x Transfer Buffer. Six 4x4" Whatman paper soaked in 1x Transfer Buffer. Transfer sandwich made in following order: cassette, mesh pad, three Whatman paper, gel, membrane with identifying pencil mark upper right corner (lay mark upper L corner on gel face down), three Whatman paper (bubbles rolled out with pipette), mesh, and cassette. Sandwich closed evenly and put in tank with membrane side to positive electrode allowing protein to move from gel to membrane. Transfer run at 85V for 60min. Membrane removed and blocked with 5% dry milk in PBS with 0.01% Tween 20 for 30 minutes at room temperature. The membrane is rinsed with PBS then washed with PBS 0.01% Tween

20 for 1 hour replacing the wash solution every 15 minutes. The primary antibody in PBS with 2% BSA and sodium azide was then applied to the membrane overnight at 4°C. Primary antibodies included laboratory p24 and Nef IgG monoclonal antibody stocks. The membrane was rinsed with PBS then washed with PBS 0.01% Tween 20 for 1 hour. Polyclonal IgG beta tubulin antibody (Abcam) in PBS with 2% BSA and sodium azide was applied to the membrane for 1 hour at room temperature. The membrane was rinsed with PBS then washed with PBS 0.01% Tween 20 for 1 hour. The secondary antibody diluted in 5% dry milk in PBS with 0.01% Tween 20 was applied to the membrane for 1 hour at room temperature. Secondary antibody included anti-mouse (p24 or Nef) or rabbit (beta tubulin) infrared-tagged monoclonal antibodies (IRDye800 anti-mouse IgG Rockland Immunochemicals, Alexa Fluor 680 anti-rabbit IgG Invitrogen). The membrane was rinsed with PBS then washed with PBS 0.01% Tween 20 for 1 hour. A final rinse with PBS was performed before the Odyssey Infrared Scanner was used to analyze the blot. Nef and p24 protein levels were normalized to cellular Beta Tubulin levels to account for differences in sample loading. The final p24 and Nef values were compared to determine Rev-RRE selective inhibition by the compounds.

Transfection of NL4-3 with wild type or mutant RREs with Rev Titration

293T cells were transfected with 5μg NL4-3 Rev (-) wild type or mutant RREs and increasing concentrations of pCMV Rev. The total level of promoter was normalized by adding empty pCMV. The wild type Rev (-) provirus was compared to the mutant RREs with the same Rev titration. p24 ELISA was used to analyze day 3 supernatant samples.

Infectivity FACS assay of NL4-3 produced in differing concentrations of Rev

NL4-3 Rev (+) or (-) produce from 293T transfection with either 35ng or 600ng Rev were used to infect GFP reporter cells in a dilution series in the presence of 100nmol Saquinavir. The cells were fixed with 2% paraformaldehyde after 48 hours. The cellular expression of GFP, measured by FACS, in each dilution was used to compare the infectivity of the different viruses. NL4-3 virus was used to infect G11 CEM cells (Rev and Tat dependent) and NL4-3 Rev (-) was used to infect Ghost cells (Tat only dependent).

Transfection of GagPol constructs with wild type or mutant RREs with Rev Titration

293T cells were transfected with 5μg pCMVGagPol and increasing concentrations of pCMV Rev. The total level of promoter was normalized by adding empty pCMV. The wild type NL4-3 RRE Rev (-) GagPol

construct was compared to the mutant RREs with the same Rev titration. p24 ELISA was used to analyze day 3 supernatant samples.

Long Term Double Selection of NL4-3 and Mutant Viruses

Viral cultures of wild type and resistant variants were performed first comparing growth in the presence of various concentrations of T-20. Then long term selection cultures were performed as described with #1272, #3539, #3543 produced virus in the presence of both T-20 and 103833 or 104366 in various concentrations (0.75 or 1ng T-20, 1μM 103833 or 0.8μM 104366). When an increase in the p24 level was observed for the long term selection cultures exposed to both compounds (indicating viral growth) the viral supernatant was used infect T cell lines in comparison to the original NL4-3 nef (-) virus. Infections were performed as previously described either in the absence of compound, in the presence of 104366 or 103833, T-20, or both T-20 and Rev-RRE inhibitor compounds (1ng T-20, 10μM 103833 or 8μM 104366). When the new viral variant was able to replicate in the presence of compounds at the concentrations tested, the resistant variant was isolated for further study. Supernatant and cell samples were collected for these cultures and stored at –80°C.

Isolation of Mutations Responsible for Resistance to Double Selection

Mutations were identified after isolation of viral RNA, amplification of segments of interest, and DNA sequencing as previously described. PCR fragments were separated using TOPO cloning. Mutations of interest were built into the pNL4-3 backbone as previously described to test for the cause of resistance to both compounds.

Long Term Selection of R73 Virus

R73 HXB2 like virus was used to test T-20 and Rev-RRE inhibitor effectiveness on a different viral background. R73 was used to infect SupT1 T cells as previously described in the absence of compound or compared to T-20, 103833, or 104366 (T-20 10ng or 100ng, 1μM or 10μM 103833, 0.8μM or 8μM 104366). Cultures were split every 3 days and evaluated for p24 levels by ELISA. The first culture was performed at the same viral input as the NL4-3 selection cultures. Then in subsequent cultures the viral input was reduced by five times. Virus was tested for resistance and characterized as previously described.

Isolation of Mutations Responsible for R73 Rev-RRE Compound Resistance

Mutations were identified after isolation of viral RNA, amplification of segments of interest, and DNA sequencing of PCR product as previously described.

Primers used for amplification

Fragment containing Rev exon 1:

379-5’ CAA TGA ATG GAC ACT AGA GC 3’

479-5’ TCC CCT CCT GAG GAT TGC 3’

Fragment containing RRE, Rev exon 2:

1557-5’ GCA CAT TGT AAC ATT AGT AGA GC 3’

1781- 5’ CTA AGA TCT GCA GGT CGA CGG A 3’

Site Mutagenesis by Overlap Extension PCR of R73 Virus

Site mutagenesis by overlap extension (SOE) PCR was used to generate mutations of interest in the R73 wild type virus.

Creation of single mutated overlapping fragments

Primers used for creation of single mutated fragments:

1. R73 mutagenesis of nt 7295 A-->C GAT to GCT (D to A):

1802-5’ cag ata gCt agc aaa tta aga gaa caa ttc gg 3’

1803-5’ cc gaa ttg ttc tct taa ttt gct aGc tat ctg 3’

2. R73 mutagenesis of nt 6402 A-->C GCA to GCC (A):

1804-5’ g aag gaa gcC acc acc act c 3’

1805-5’ g agt ggt ggt Ggc ttc ctt c 3’

Reaction conditions: 10x PCR buffer 5μl, $MgCl_2$ 3μl, dH_20 33.5μl, dNTP 1μl, Taq Pol 0.5μl, Primers 1μl each, R73 plasmid 5μl (50-100ng) cycled

at 95°C for 2 minutes, (95°C for 1 minute, 45°C for 1 minute, 72°C for 2 minutes) for 30 cycles, then 72°C for 10minutes and storage at 4°C.

Using the Qiagen PCR minielute kit the PCR products were purified prior to the next reaction.

Creation of large fragment with single mutation of interest

Primers for creation of large fragments joined due to overlap extension of mutated areas:

Outer fusion PCR primers including NcoI and BsaB1 sites:

379-5' CAA TGA ATG GAC ACT AGA GC 3'

1808-5' CAA GTC TGA AGA TCT CGG AC 3'

Reaction conditions: 10x PCR buffer 5μl, $MgCl_2$ 3μl, dH_20 28.5μl, dNTP 1μl, Taq Pol 0.5μl, Primers 1μl each, Purified PCR reactions with smaller overlapping fragments 5μl each cycled at 95°C for 2 minutes, (95°C for 1 minute, 45°C for 1 minute, 72°C for 2 minutes) for 30 cycles, then 72°C for 10minutes and storage at 4°C.

PCR product was run on 1% agarose gel in 1xTAE and appropriate bands extracted with Qiagen gel extraction kit per manufacturers instructions.

Creation of double mutant overlapping fragments

Primers for creation of additional mutation in large fragments from previous SOE PCR:

1. R73 mutagenesis of nt 7295 A-->C GAT to GCT (D to A) used on large fragment containing mutation at nt 6402:

1802-5' cag ata gCt agc aaa tta aga gaa caa ttc gg 3'

1803-5’ cc gaa ttg ttc tct taa ttt gct aGc tat ctg 3’

2. R73 mutagenesis of nt 6402 A-->C GCA to GCC (A) used on large fragment containing mutation at nt 7295:

1804-5’ g aag gaa gcC acc acc act c 3’

1805-5’ g agt ggt ggt Ggc ttc ctt c 3’

Reaction conditions: 10x PCR buffer 5μl, $MgCl_2$ 3μl, dH_20 33.5μl, dNTP 1μl, Taq Pol 0.5μl, Primers 1μl each, Large fragment PCR product containing single mutation cycled at 95°C for 2 minutes, (95°C for 1 minute, 45°C for 1 minute, 72°C for 2 minutes) for 30 cycles, then 72°C for 10minutes and storage at 4°C.

Using the Qiagen PCR minielute kit the PCR products were purified prior to the next reaction.

Creation of large fragment containing double mutations

Primers for creation of large fragments joined due to overlap extension of mutated areas:

Outer fusion PCR primers including NcoI and BsaB1 sites:

379-5’ CAA TGA ATG GAC ACT AGA GC 3’

1808-5’ CAA GTC TGA AGA TCT CGG AC 3’

Reaction conditions: 10x PCR buffer 5μl, $MgCl_2$ 3μl, dH_20 28.5μl, dNTP 1μl, Taq Pol 0.5μl, Primers 1μl each, Purified PCR reactions with overlapping double mutant fragments 5μl each cycled at 95°C for 2 minutes, (95°C for 1 minute, 45°C for 1 minute, 72°C for 2 minutes) for 30 cycles, then 72°C for 10minutes and storage at 4°C.

PCR product was run on 1% agarose gel in 1xTAE and appropriate bands extracted with Qiagen gel extraction kit per manufacturers instructions.

Creation of Mutant R73 Proviruses

Large SOE fragments and R73 parent provirus were digested sequentially with BsaB1 in NEB buffer 2 at 60°C for 2 hours, then NcoI at 37°C for an additional 2 hours. The digests were run on 1% agarose gel in 1xTAE and the appropriate bands extracted as previously described. Mutated fragments were ligated into the R73 vector using T4 DNA ligation as previously described. The resultant proviruses were purified by gel extraction then used to transfect 293T cells to produce virus. Mutational changes were confirmed by DNA sequencing.

Growth of R73 Mutants in SupT1 cells

R73 mutant viruses were used to infect SupT1 cells and grown in the presence of 103833 or 104366 compared to wild type R73. The cultures were split every 3 days as previously described. In house ELISA was used to measure p24 values.

R73 Rev (-) Site mutagenesis by Overlap Extension PCR

Creation of R73 Rev (-) overlapping fragments

Primers used for creation of Rev (-) R73:

R73 Rev exon 1 aa 23 mutagenesis TAT to TAA (premature stop):

1806-5’ c aag ctt ctc taA caa agc ag 3’

1807-5’ ct gct ttg Tta gag aag ctt g 3’

Reaction conditions: 10x PCR buffer 5μl, $MgCl_2$ 3μl, dH_20 33.5μl, dNTP 1μl, Taq Pol 0.5μl, Primers 1μl each, R73 plasmid 5μl (50-100ng) cycled at 95°C for 2 minutes, (95°C for 1 minute, 45°C for 1 minute, 72°C for 2 minutes) for 30 cycles, then 72°C for 10minutes and storage at 4°C.

Using the Qiagen PCR minielute kit the PCR products were purified prior to the next reaction.

Creation of large fragment with single mutation of interest

Primers for creation of large fragments joined due to overlap extension of mutated areas:

Outer fusion PCR primers including NcoI and BsaB1 sites:

379-5’ CAA TGA ATG GAC ACT AGA GC 3’

1808-5’ CAA GTC TGA AGA TCT CGG AC 3’

Reaction conditions: 10x PCR buffer 5μl, $MgCl_2$ 3μl, dH_20 28.5μl, dNTP 1μl, Taq Pol 0.5μl, Primers 1μl each, Purified PCR reactions with smaller overlapping fragments 5μl each cycled at 95°C for 2 minutes, (95°C for 1 minute, 45°C for 1 minute, 72°C for 2 minutes) for 30 cycles, then 72°C for 10minutes and storage at 4°C.

PCR product was run on 1% agarose gel in 1xTAE and appropriate bands extracted with Qiagen gel extraction kit per manufacturers instructions.

Transfection of 293T with R73 Rev (-) and NL4-3 Rev (-) with Rev Titration

293T cells were transfected with 5μg provirus and increasing concentrations of pCMV Rev by $CaPO_4$ method. The total level of promoter was normalized by adding empty pCMV. R73 Rev (-) (lacking vpu, vpr, nef) was compared to NL4-3 Rev (-) with the same Rev titration. p24 ELISA was used to analyze day 3 supernatant samples.

Western Blot comparing 1st round steady state Env levels of Viruses

NL4-3, R73, Mutant Viruses were cultured in SupT1 cells in the presence of 100nmol Saquinavir. Cells collected at day three of the first round cultures were used for western blots for p24, p55, and Env. Western blots were performed as previously described. Primary antibodies consisted of p24 and Env IgG monoclonal laboratory stocks. The Odyssey infrared scanner was used to measure blots. The proteins were normalized to Beta Tubulin and p55. Steady state Env levels were compared between the different viruses.

Chapter 3

Results

Background Research

Our laboratory through cooperative efforts with Southern Research Institute and Message Pharmaceuticals has identified small molecule inhibitors of HIV-1 viral replication. The compounds are small heterocyclic molecules with differing chemical structures. Previous work done with these compounds is the foundation for this research. Figures 6, 7, 9, 10, 11, 13, 14, and Tables 1, 2 show the work performed by collaborators and do not reflect my research data. Because these results set up the background for my research I included the data within this dissertation.

Cell-Based Screen Assay for Post-Integration HIV Life Cycle

Our laboratory developed a cell line that constitutively expresses high levels of the HIV-1 structural proteins in a Rev-dependent fashion (153). This cell line (5BD.1) excretes about 10-15ng of p24/ml into the media over a 24 hour period in the form of virus like particles. Transfection studies, using a HIV vector have demonstrated that the particles are infectious.

We reasoned that the cell line could be used to screen for compounds that inhibited post-integration steps in the HIV life cycle, in particular Rev function and particle assembly and release. Addition of a compound to the cells, which inhibited a critical post integration step in the

HIV life cycle, would cause a decrease in particle production. This could easily be quantified by measuring p24 levels in the medium.

Using this cell line, 40,000 compounds from a library supplied by Specs B.V. (Delft, the Netherlands) were screened at Message Pharmaceuticals (Malvern, PA until 2004), in an assay using a 384 microtiter plate format at a final compound concentration of 10μM in 0.001% DMSO. To promote the identification of compounds that might be inhibitors of Rev function we carried out a two step compound addition procedure, where compound was added for 16hrs, washed off with the medium, then re-added. After an additional 8 hours of incubation, media was collected and assayed for p24. Since Rev acts at an upstream step in the gene expression-particle assembly pathway, this two step drug addition procedure, which discarded the p24 produced during the first 16 hours of drug addition, should allow Rev inhibitors to score in the assay. Figure 6 shows the p24 values obtained for 5120 of the 40,000 compounds, expressed as a percentage of the control value. Compounds that inhibited p24 production by more than 50% were retained for further study. In this batch thirteen compounds gave p24 values that were less than 50% of the control. In total, 192 compounds, from the 40,000 screened, caused greater than 50% inhibition of p24.

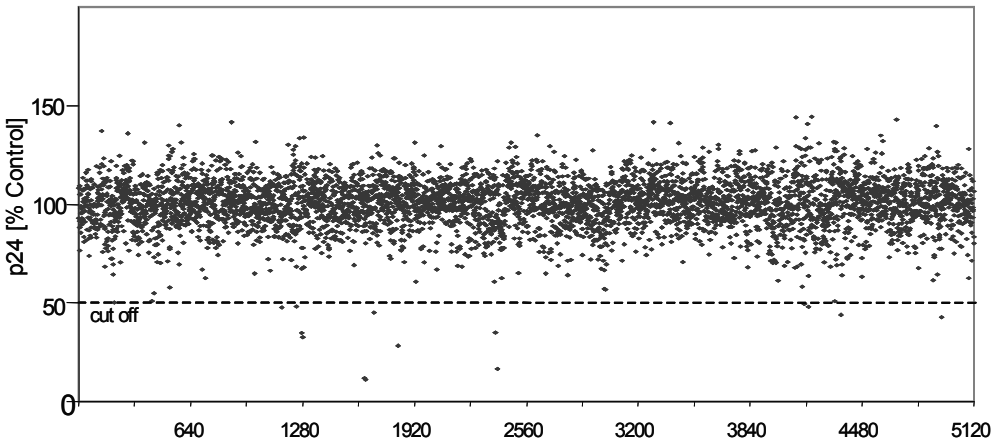


Figure 6. Primary Screening Assay in 5BD.1 cells.

5BD.1 cells constitutively producing HIV viral like particles in a Rev dependent manner were used to test the initial 40,000 heterocyclic compounds. p24 production was measured by ELISA to compare viral particle production from cells exposed to compound to those exposed only to media and DMSO (control). 5120 of the compounds are demonstrated with the thirteen points below the 50% cut off line indicating compounds that were selected for further testing. 192 of the original total compounds were selected in this way and analyzed further in dose response and toxicity assays.

Each of the 192 compounds was further screened for specificity and toxicity in six point dose response assays, using the same cell line and an additional cell line (MT4) for a second toxicity assay. To perform the dose response assays, compounds were added to 5BD.1 cells that were plated into 96 well plates at 20,000 cells/ml. The plates were incubated in the presence of varying concentrations (30μM, 10μM, 3μM, 1μM, 0.3μM, 0.1μM, or 0 for controls) of the 192 compounds for 16 hours. After 16 hours the media was removed and discarded. 135μl of fresh media with the appropriate concentration of compound was applied and the cells were incubated for an additional 24 hours. Supernatant was collected and used in a p24 ELISA.

For the toxicity assay in 5BD.1 cells, cells were collected after 40hrs and subjected to the Promega CellTiter One Solution Cell Proliferation Assay (Promega, Madison, WI). The bioreduction of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)), which is a measure of cells that have metabolically active dehydrogenases, gives a proportional measure of viable cells. At all concentrations tested, the compounds showed little toxicity in the 5BD.1 cell line. To further examine the toxicity of the compounds in a cell line that is infectable by HIV, compounds were tested in the MT4 cell line after 5 days of incubation. In this case, both compounds showed little toxicity up to 10μM, after which toxicity was readily apparent. Figure 7 panels A and B show the efficacy and toxicity dose response assays for

the two of the compounds, 103833 and 104366 (code name generated by Message Pharmaceuticals) in 5BD.1 cells. Very little toxicity was observed in these cells. Both compounds showed a good linear response up to concentrations of 10μM. The toxicity results for both compounds in the more sensitive MT4 cells are shown in panel C of Figure 7.

In total, 12 compounds emerged as candidates. The IC50 and TC50 values for these 12 compounds in the dose response and toxicity assays are listed in Table 1. The IC50 represents the dose of the compounds that inhibited the p24 level produced by the 5BD.1 cells to below 50% compared to the control. The TC50 values indicate the dose of the compounds that caused toxicity in 50% or more of the cells compared to the control in both the 5BD.1 cells and MT4 cells. The therapeutic index of the compounds is derived from these values (TC50/IC50). Figure 8 illustrates the structures of these compounds.

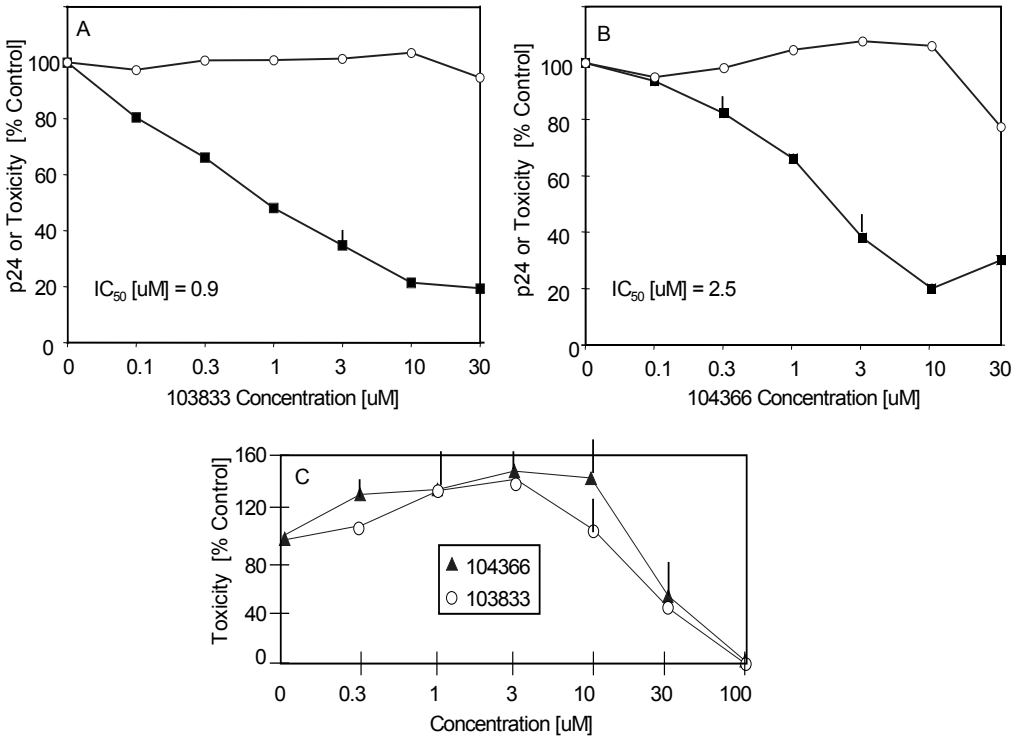


Figure 7. Dose Response and Toxicity Assays for 103833 and 104366.

A: Six concentrations of 103833 were tested in 5BD.1 cells for inhibition of p24 and cellular toxicity compared to control. IC50 calculated for assay=0.9μM, TC50>30μM. p24 (■) Toxicity (o)

B: Six concentrations of 104366 were tested in 5BD.1 cells for inhibition of p24 and cellular toxicity compared to control. IC50 calculated for assay=2.5μM, TC50>30μM. p24 (■) Toxicity (o)

C: Six concentrations of 103833 and 104366 were tested using MTS Toxicity assay in MT4 cells compared to control. TC50 calculated for 103833=32μM. TC50 calculated for 104366=28μM.

ID Number	p24 IC50 (μM) (5BD.1)*	TC50 (μM) (5BD.1)*	TC50 (μM) (MT4)**
74377	22	>30	>100
74378	30	>30	>100
73497	10	>30	>100
75168	3	>30	>100
89246	0.9	>30	20
91161	1.9	25	18
103833	0.9	>30	32
104366	2.5	>30	28
106904	3.2	>30	50
107129	0.2	>30	10
107740	4.9	>30	50
109020	3	>30	100

* Measured after 2 days
**Measured after 5 days

Table 1: IC_{50} and TC_{50} of Compounds Selected From The Dose Response And Toxicity Assays.

After the primary screening 192 compounds were tested in Dose Response and Toxicity Assays. Twelve Compounds were chosen based on the IC50 and TC50 values that determine a favorable therapeutic index (TC50/IC50). ID numbers for the twelve compounds (assigned in primary screen by Message Pharmaceuticals) are shown in the table corresponding to the IC50 and TC50 values. IC50 values indicate the concentration of the compounds that inhibited the p24 level produced by the 5BD.1 cells to below 50% compared to the control. The TC50 values indicate the concentration of the compounds that caused toxicity in 50% or more of the cells compared to the control in both the 5BD.1 cells and MT4 cells.

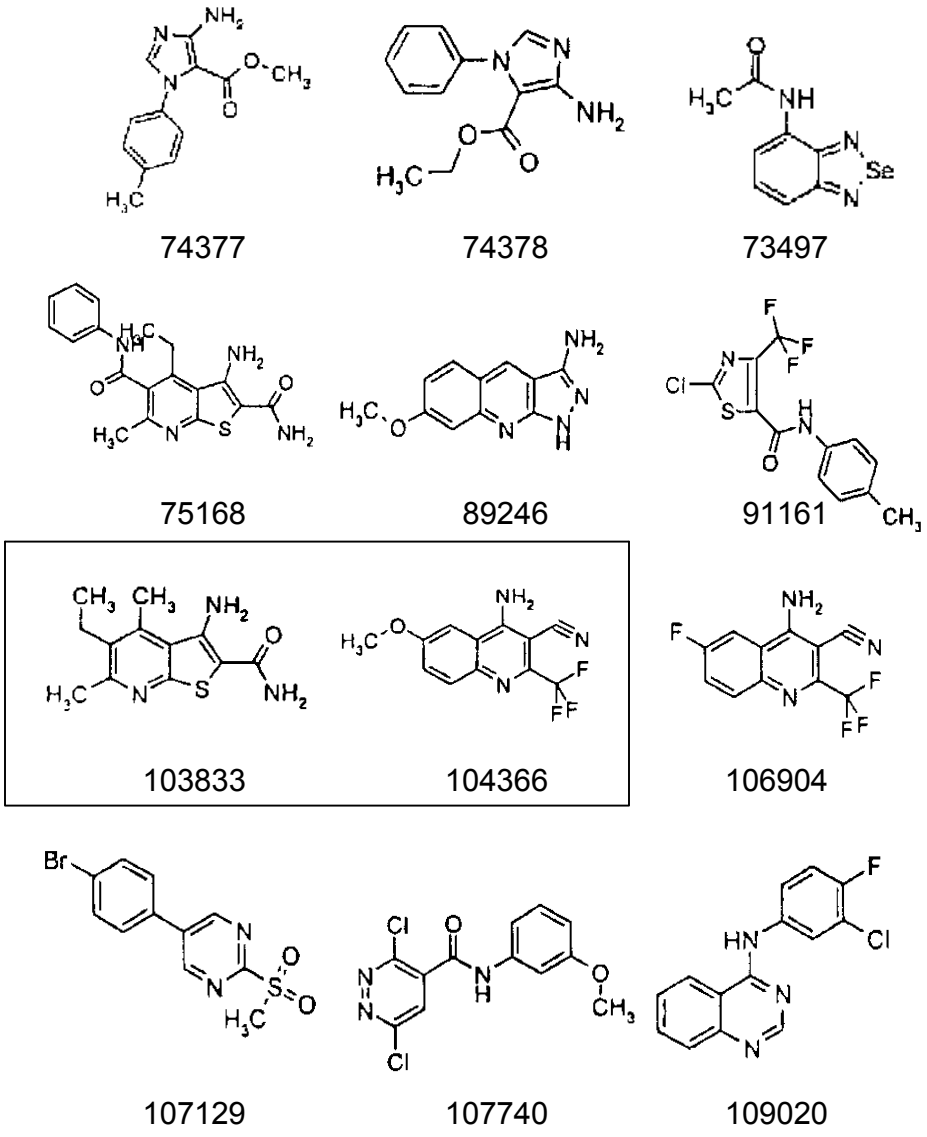


Figure 8. Structures of 12 Heterocyclic Compounds Selected from Dose Response and Toxicity Assays. Compounds 103833 and 104366 are annotated with box.

Cell Based Assay for Rev Function

Since our laboratory has a longstanding interest in HIV Rev function, we next tested the compounds in an assay that was designed to measure inhibition of Rev activity. This assay utilizes HeLa derived cells containing a stably integrated, well-established, Rev-reporter construct. The construct is a variation of a reporter plasmid that expresses the chloramphenicol acetyl-transferase (CAT) gene in a Rev dependent context from the CMV immediate early promoter, in that the construct used in this assay contains Renilla Luciferase substituted for the CAT gene. Renilla Luciferase is an enzyme that can be more easily measured than CAT. The cell line also contains a second Firefly Luciferase reporter that serves as the “toxicity” control. This reporter is expressed from a stably integrated bicistronic expression construct that also expresses the HIV-1 IIIB Rev gene from an IRES. Expression of these proteins is under the control of a single Tet-Off promoter.

To perform this assay, doxycycline is removed from the cells, as compound is added, and the two luciferases are measured after 24 hours of incubation at 37°C. If a drug candidate were a specific inhibitor of Rev function, it would be expected to decrease the amount of Renilla Luciferase, but not Firefly Luciferase.

The compounds were shown to selectively inhibit the Renilla luciferase reporter in a dose dependent manner (Figure 9). For 103833

the shape of the inhibition curve for the Firefly Luciferase is not understood, but even with the slight inhibitory effect seen, a clear difference in the responses of the Renilla (Rev-dependent) and Firefly Luciferase (Rev-independent) inhibition was observed. 104366 showed virtually no inhibition of the Firefly Luciferase, and a linear dose response between 0.8μM and 7.9μM for the Renilla Luciferase. As a control Leptomycin B was also tested (Figure 9). This is a known inhibitor of HIV Rev function, since it is known to bind to and inhibit the Rev co-factor Crm1. The compound is also quite toxic to cells and causes a dramatic decrease in both reporters at the highest concentration tested. However, at 10nM a clear differential between the two luciferases can be observed, confirming the specificity of the assay (Figure 9). 103833 and 104366 scored as potential specific inhibitors of Rev function and were used further in this research. Both compounds gave IC50 values lower than 2.5μM in the 5BD.1 cell assay, with at least one log difference in toxicity. Figure 8 illustrates the chemical structures of both these compounds.

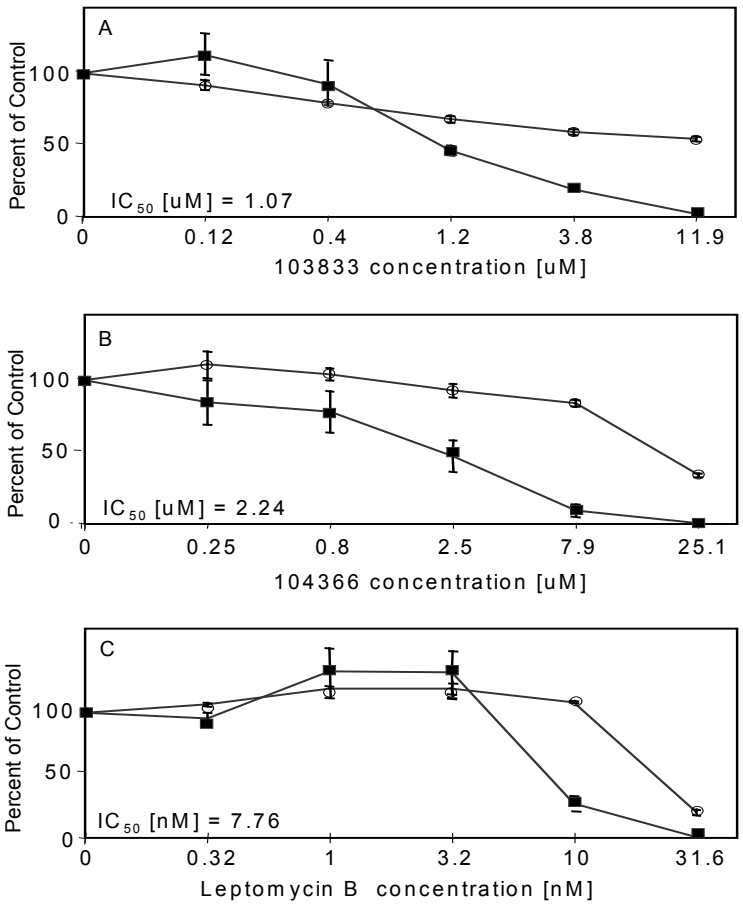


Figure 9. Dose Response Assay for 103833 and 104366 Comparing Effect on Rev Dependent and Rev Independent Luciferases.

HeLa cells containing stably integrated expression plasmids for Rev-dependent Renilla Luciferase (■) or Rev-independent Firefly Luciferase (o) were treated with concentrations of compounds. Curves represent level of reporters expression at 24 hours at the concentrations tested compared to a control with no drug added. A: 103833 IC50 of Renilla Luciferase=1.07μM. B: 104366 IC50 of Renilla Luciferase=2.24μM. C: Leptomycin B hCRM1 inhibitor IC50=7.76nM

Compound Specificity for Rev-RRE Using CTE Control

HIV-1 uses the Rev-RRE pathway to mediate export of unspliced and incompletely spliced mRNA. Other retroviruses use different mechanisms to accomplish the same task. For example, Mason Pfizer Monkey Virus uses a cis-acting RNA element the Constitutive Transport Element (CTE) that interacts with the cellular protein Tap to mediate export. The Tap pathway works independently of the Rev cofactor Crm1, and is not sensitive to inhibition by Leptomycin B. To determine if the compounds 103833 and 104366 specifically inhibit the Rev-RRE pathway, we tested the compounds in transient transfections of 293T cells using vectors containing either pCMVGagPol-RRE and pCMVRev, or pCMVGagPol-CTE. The vectors produce HIV pseudovirions (measured by p24 ELISA) by their respective pathways (Rev-RRE or CTE). Figure 10 shows the dose response curves for each transfection. After 72hrs the p24 production mediated by the Rev-RRE pathway was sensitive to each compound in a dose dependent manner, and importantly the IC50 values were in the same low μM range as previous assays.

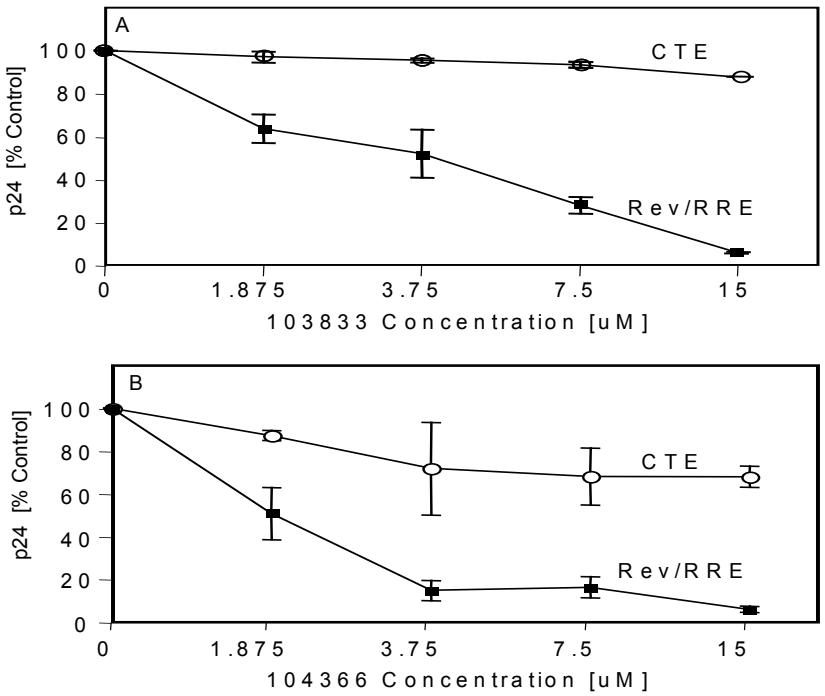


Figure 10. Dose Response Assays for 103833 and 104366 Comparing Effect on p24 Expression from Rev-RRE Dependent construct compared to MPMV CTE construct.

293T cells were transfected with either pCMVGagPol-RRE and pCMV Rev (■), or pCMVGagPol-MPMV CTE (O). RRE is Rev-dependent. CTE is Rev-independent. The p24 production of the constructs at 72 hours, compared to a control with no added drug, was used to determine the inhibition by the compounds at the concentrations tested. A: results for 103833. B: results for 104366.

U1 Latency Assay

To further examine the specificity of the compounds for the Rev-RRE pathway the compounds were tested in U1 cells at Southern Research Institute. U1 histiocytic leukemia cells contain an integrated HIV-1 provirus that can be induced by TNF-alpha or PMA to start viral replication (34, 38, 63, 64). After induction, the cells express the entire post-integration phase of the virus-life cycle. This is where the Rev-RRE pathway is known to function. To perform the assay, cells were induced with TNF-alpha, compound was added at various concentrations, and virus production was monitored by measuring reverse transcriptase released into the medium after 72 hours. Temacrazine, a known inhibitor of HIV transcription, was used as a control compound. Figure 11 shows the results of this assay. Both compounds effectively inhibited HIV particle production in a dose dependent fashion. Again the inhibition was in approximately the same concentration range where inhibition was seen in the other assays.

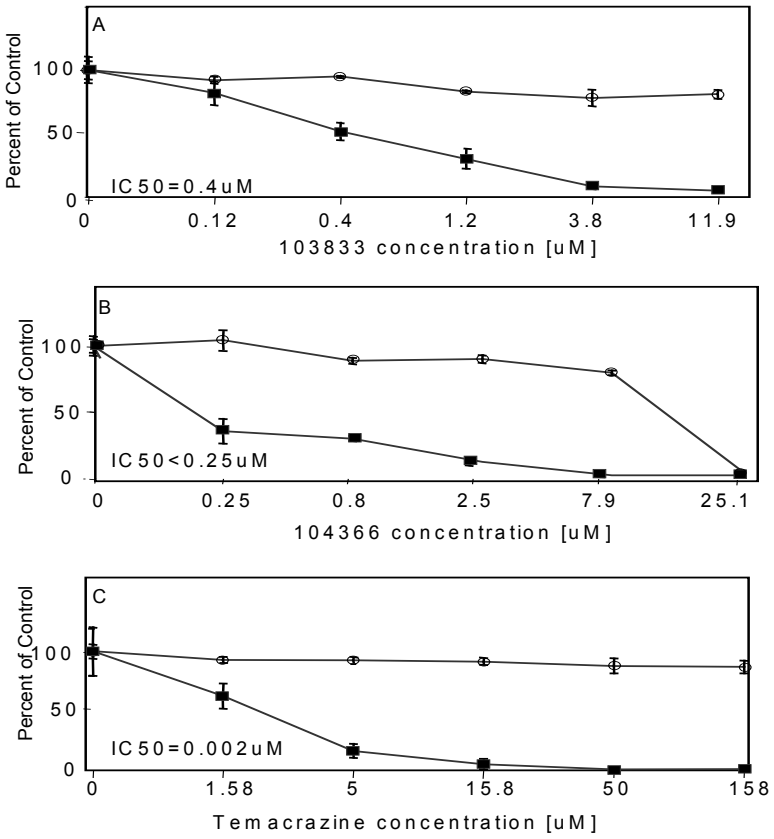


Figure 11. Dose Response Assay for 103833 and 104366 in U1 Cells. TNF-alpha induced U1 cells viral production in the presence of the compounds at the concentrations tested. Viral release from cells was measured as reverse transcriptase activity in the medium. IC50 represents the concentration of the compounds that inhibited reverse transcriptase activity below 50% compared to the control with no added drug (Reverse Transcriptase ■). Toxicity was measured with MTS dye for cell viability as previously described in methods (Toxicity O). A: 103833 IC50=0.4μM. B: 104366 IC50<0.25μM. C: Temacrazine LTR inhibitor IC50=0.002μM.

Specificity of Compounds for Rev-dependent Proteins

The Dual Luciferase Assay and Transfection Assay with the CTE control had already shown specificity of the compounds for the Rev-RRE pathway. We reasoned that analysis of the viral protein products produced from the U1 cells released from latency, in the presence of the compounds, would be another way to determine if the compounds were specific for functional inhibition of Rev. This is because Rev function is only required for export of the unspliced and incompletely spliced HIV mRNA, not mRNAs that are fully spliced. Thus inhibition of Rev function would be expected to have little or no effect on the Nef protein production, because it is made from a fully spliced mRNA. But it should inhibit p55 and p24 synthesis produced from unspliced mRNA that is completely Rev dependent.

To further investigate specificity, western blots of both the cells from the previously described U1 latency assay, and 293T Transfections with NL4-3 in the presence of the compounds (see methods), were performed as described in the methods measuring the p24 versus Nef protein levels. Blots were quantitated using a fluorescence scanner (Odyssey, LiCor Inc.). In both assays the p24 was selectively reduced compared to Nef that was largely unchanged. The blots also showed selective reduction of p55 Gag (Rev-dependent). The results overall demonstrate selectivity of the compounds for the Rev-RRE pathway (Figure 12).

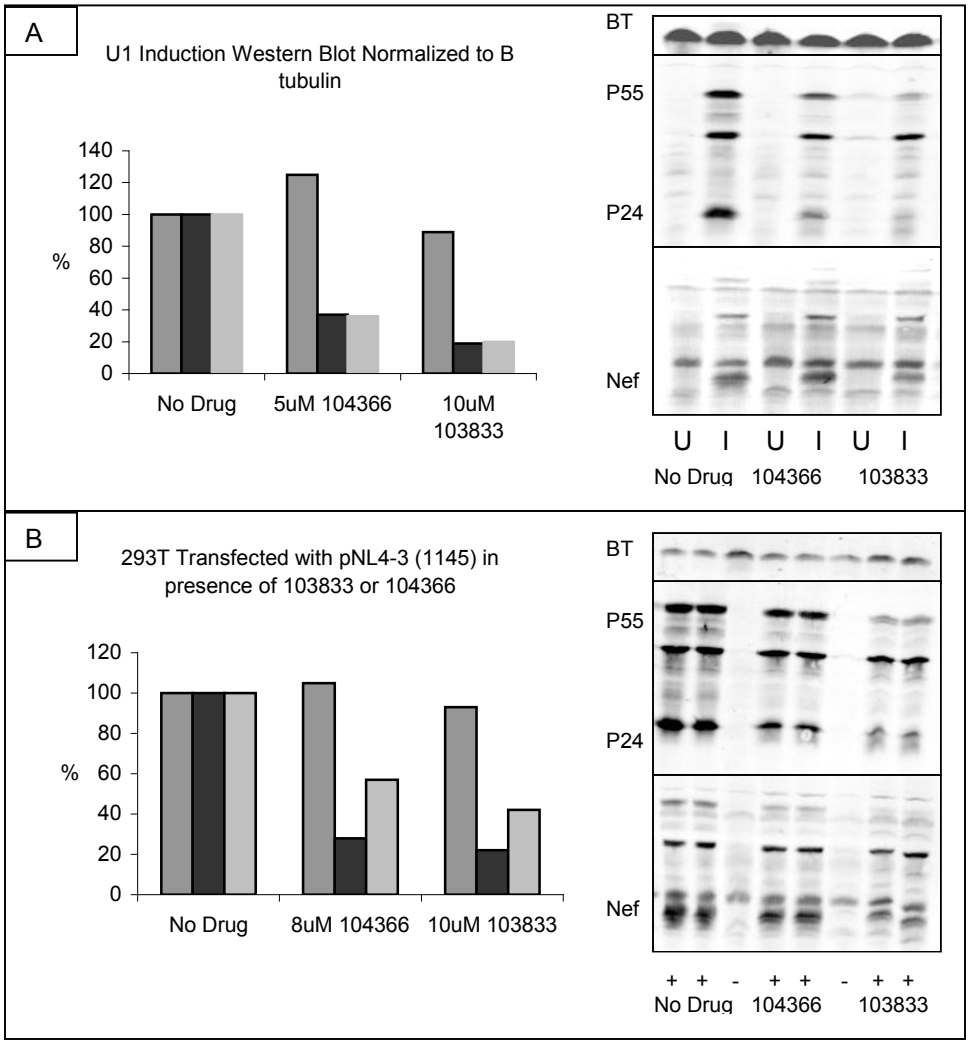


Figure 12. Specificity of Compounds for Rev Dependent Proteins.

Blots shown on right (Top to bottom: Beta Tubulin, p55 and p24, Nef). Quantification of Nef (dark gray), p24 (black), and p55 (light gray) levels, normalized to Beta tubulin, shown on left. A: U1 Cells Uninduced (U) or Induced with TNF-alpha (I) in no drug, 5μM 104366, or 10μM 103833. B: 293T cells Transfected with NL4-3 provirus #1145 (+) in no drug, 8μM 104366, or 10μM 103833. Untransfected 293Ts (-).

Rev-RRE Binding Assay

The total accumulated data presented above strongly suggest that both 103833 and 104366 work by interfering with Rev function. An early step in the RNA export pathway that is mediated by Rev is Rev-RRE binding. This step can be studied in vitro with bacterially synthesized purified Rev protein and a radiolabeled in vitro transcribed RRE RNA probe, using an agarose gel shift assay. To assess whether the compounds were capable of inhibiting this interaction, we first determined Rev-RRE binding conditions that lead to a significant shift of the radiolabeled probe (data not shown). The gel shift assay was then performed by incubating Rev, the radiolabeled probe, and compound together on ice for 30 minutes. Neomycin was included as a control since it is known to inhibit Rev-RRE interaction. The complexes that formed were then analyzed by agarose gel electrophoresis. The gel was dried and scanned using a phosphoimager (Figure 13). The data shows that the Neomycin control completely prevented the Rev-RRE complexes from forming, but neither 103833 nor 104366 had any effect on complex formation, even up to concentrations of 100μM. Thus, under the conditions tested, neither compound appears to affect Rev-RRE binding.

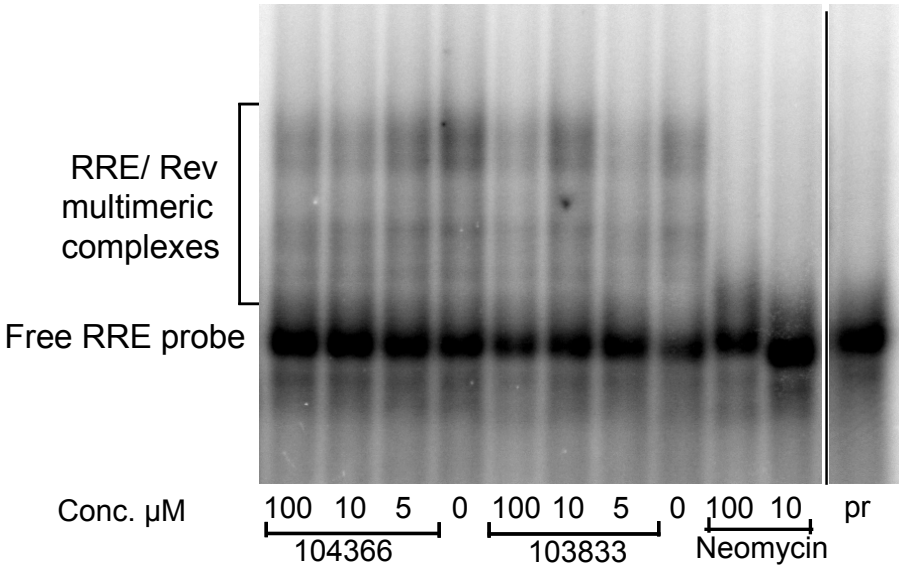


Figure 13. Dose Response Assay Using In Vitro Rev-RRE Gel Shift.

The in vitro gel shift assay was performed by incubating radiolabeled probe and bacterial Rev protein, plus or minus the indicated compounds at the indicated concentrations on ice for 10 minutes. The resulting complexes were analyzed by electrophoresis on native agarose gel. The gel was dried and examined using a phosphoimager. The lane marked (pr) contained the radiolabeled probe without added Rev protein. Neomycin, a known competitive Rev-RRE binding inhibitor, is shown at concentrations tested as a control.

Effect of Compounds on HIV Replication

We directly examined the effect of 103833 and 104366 on HIV-1 replication. At Southern Research Institute the compounds were assayed in human PBMCs and shown to inhibit viral replication of both HIV-1 NL4-3 (CXCR4 tropic Subtype B) and HIV-1 BR/93/021 (CCR5 tropic Subtype B) (44, 59) (Table 2). The viral yield produced in the presence of different drug concentrations was measured by assaying the amount of reverse transcriptase that was secreted into the media after a seven day period. The inhibition by the reverse transcriptase inhibitor AZT is shown as a control. The data from the PBMC replication assays shows clearly that both compounds inhibited the replication of the Brazilian virus with IC50 values that were similar to the values of the other assays (Figure 14). (NL4-3 curves not shown).

Growth of the laboratory isolate NL4-3 was assayed in our laboratory over a 30 day period, using a standard replication assay in which the virus was passaged in the presence of 1μM and 10μm of 103833, 0.8μM and 8μM of 104366, or in the absence of drug. Growth of NL4-3 was greatly inhibited at both concentrations tested (Figure 15).

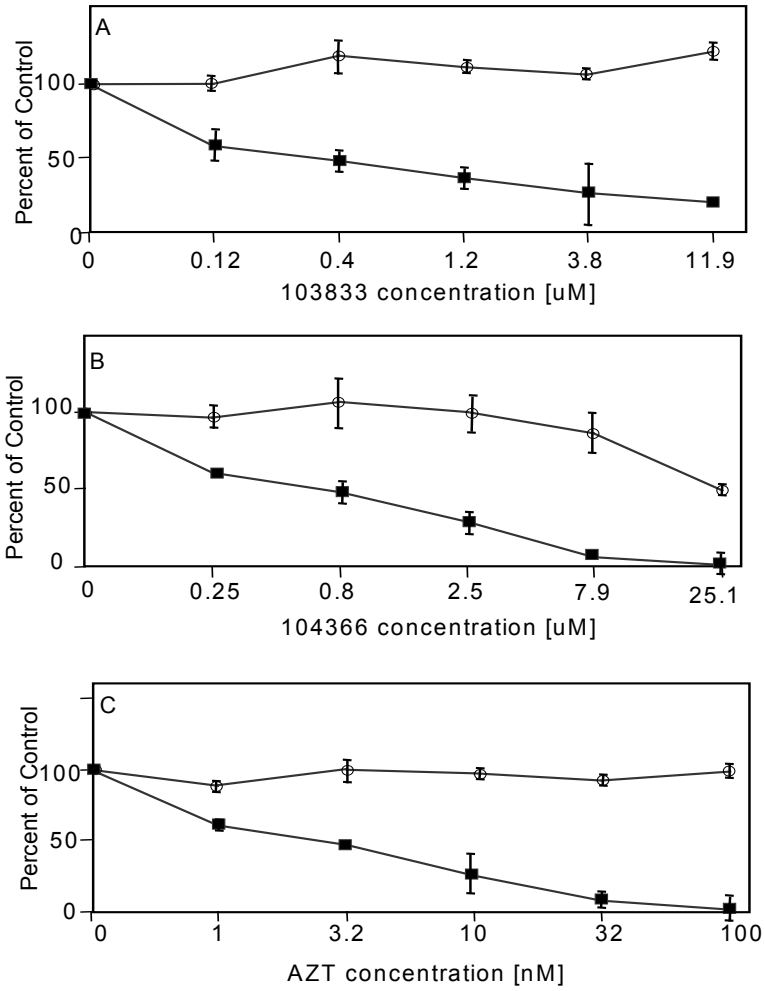


Figure 14. Dose Response Assay for Viral Replication in PBMCs.

BR/93/021 (Subtype B CRR5 tropic) HIV-1 patient isolate replication in PHA-stimulated PBMCs in the presence of inhibitor compounds. Reverse transcriptase activity measured after seven days and compared to control exposed to no drug. A: inhibition of the virus by 103833 at the concentrations tested compared to the toxicity of the compound in the MTS assay. B: Same as in A for 104366. C: Same as in A for AZT Reverse Transcriptase Inhibitor. Reverse Transcriptase (■) Toxicity (o)

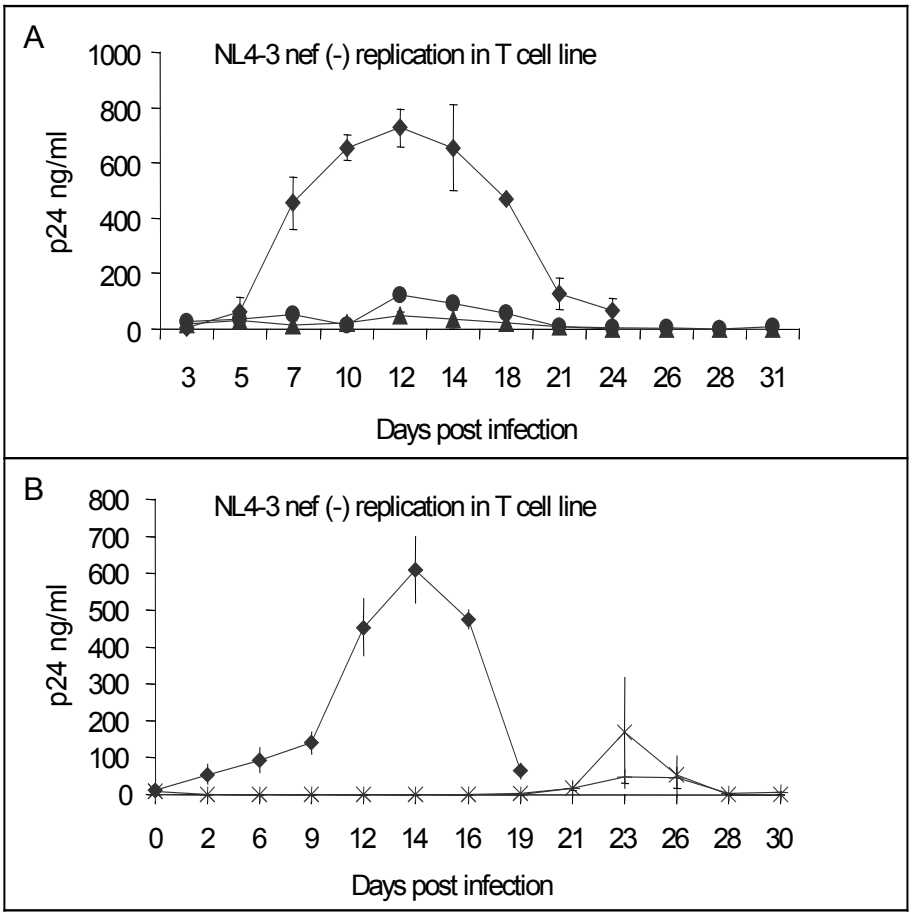


Figure 15. Virus Growth Inhibition Assay.

CEM G11 or SupT1 cells (1 million) were infected with NL4-3 virus (50ng of p24) in the presence of compound. The cultures were passaged for 30-31 days by removing three fifths of the culture on the indicated days and replacing it with fresh medium plus the concentration of compound tested. Virus yield was determined by measuring p24 in the media using ELISA. A: no drug (◆), 0.8μM 104366 (●), 8μM 104366 (▲). B: no drug (◆), 1μM 103833 (×), 10μM 103833 (+).

Inhibition of Viral Growth by compounds 103833 and 104366 is reversible

We performed an experiment to determine whether the inhibition of viral growth that was observed in cultures containing the compounds could be reversed when the compound was removed. To do this SupT1 cells were infected with virus in the presence of either 1μM or 10μM of compound 103833, or 0.8μM or 8μM of compound 104366. The compound was removed from the culture after 23 days, and was continued for an additional 13 days. The results are shown in Figure 16. In all cases, a peak of viral growth appears in each of the cultures that had compound removed about 4 days after the removal. This shows clearly that the compounds did not damage the cells in any irreversible way, nor did they eliminate the virus completely from the culture.

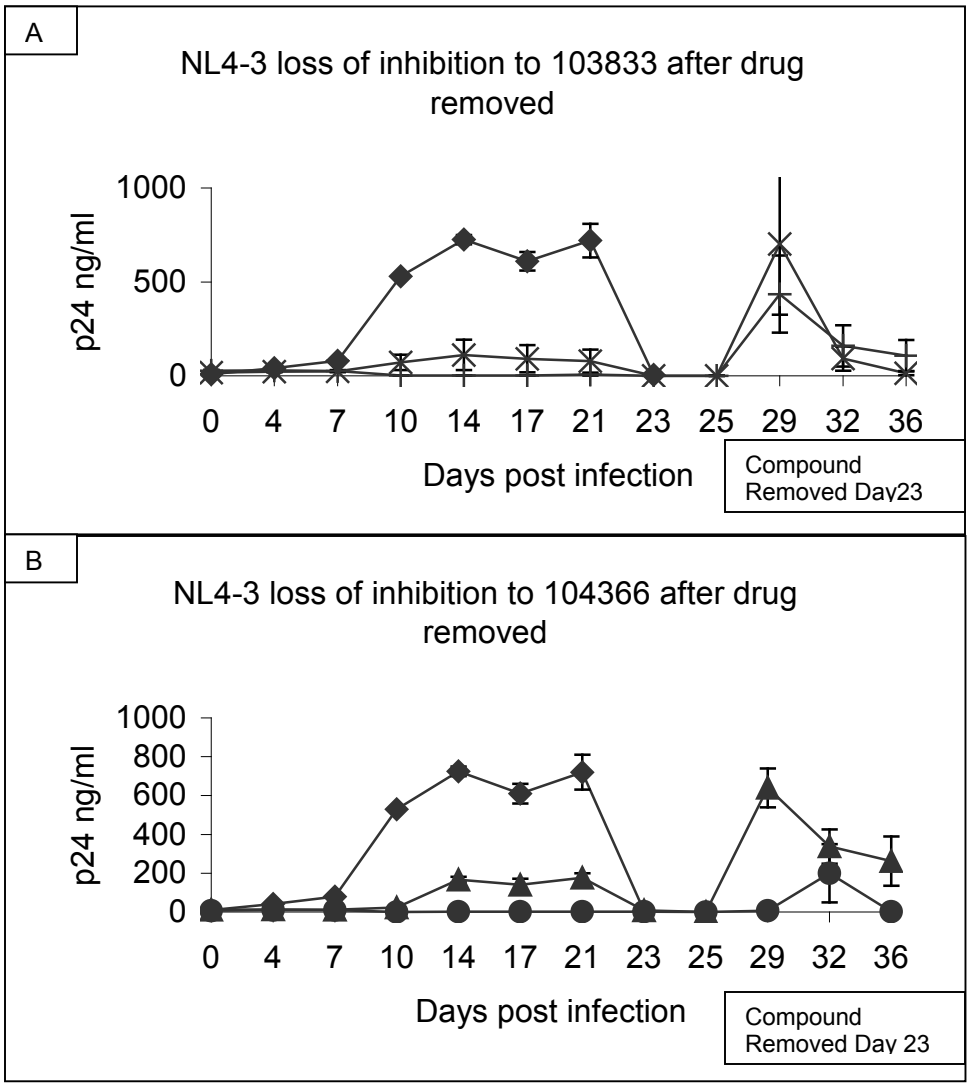

A
NL4-3 loss of inhibition to 103833 after drug removed
p24 ng/ml
1000
500
0
0 4 7 10 14 17 21 23 25 29 32 36
Days post infection
Compound Removed Day23
B
NL4-3 loss of inhibition to 104366 after drug removed
p24 ng/ml
1000
800
600
400
200
0
0 4 7 10 14 17 21 23 25 29 32 36
Days post infection
Compound Removed Day 23

Figure 16. NL4-3 growth is inhibited in a reversible manner by compounds 103866 and 104366.

A: SupT1 cells were infected with NL4-3 (50ng p24) in the presence of no compound (◆), 1μM 103833 (X), or 10μM 103833 (+). The compounds were removed on day 23. Each curve represents duplicate samples with error bars shown. p24 released into the medium was measured at each time point indicated. The data plotted is the average of the duplicate cultures with error bars shown.

B: SupT1 cells were infected with NL4-3 (50ng p24) in the presence of no compound (◆), 0.8μM 104366 (▲), or 8μM 104366 (●). The compounds were removed on day 23. Each curve represents duplicate samples with error bars shown. p24 released into the medium was measured at each time point indicated. The data plotted is the average of the duplicate cultures with error bars shown.

Long Term Selection of Rev-RRE Inhibitor Resistant Variants

Selection of resistance to compound 104366

In order to further characterize the action of the small molecule inhibitors on the Rev-RRE pathway, we carried out long term infection of HIV-1 NL4-3 nef (-) virus in G11 cells in the presence of 0.8μM 104366 (IC50 of compound), as described in the methods, with the purpose of selecting resistant variants. Duplicate cultures were infected. Continued passage in the presence of 0.8μM 104366 largely suppressed viral replication until 47 days, when one culture showed growth (Figure 17).

Virus from Day 53 of the infection was used to infect SupT1 cells in the presence of the same concentration of compound 104366 and also a 10 fold higher concentration, to determine if it was truly a resistant variant that had been selected. As a control, the parental virus was also retested (Figure 18). Those cultures demonstrated that the compound largely repressed the wild type parent virus, while the resistant variant replicated at all concentrations tested. The variant also showed increased fitness by the ability to reach peak viral growth before the wild type parent.

This resistant virus was then tested for cross resistance to compound 103833. To do this SupT1 cells were infected in the presence of 1μM or 10μM 103833 with the Day 53 virus. For a direct comparison virus was also grown again in the presence of 104366 (Figure 19). The results show that overall the Day 53 virus is resistant to both compounds tested.

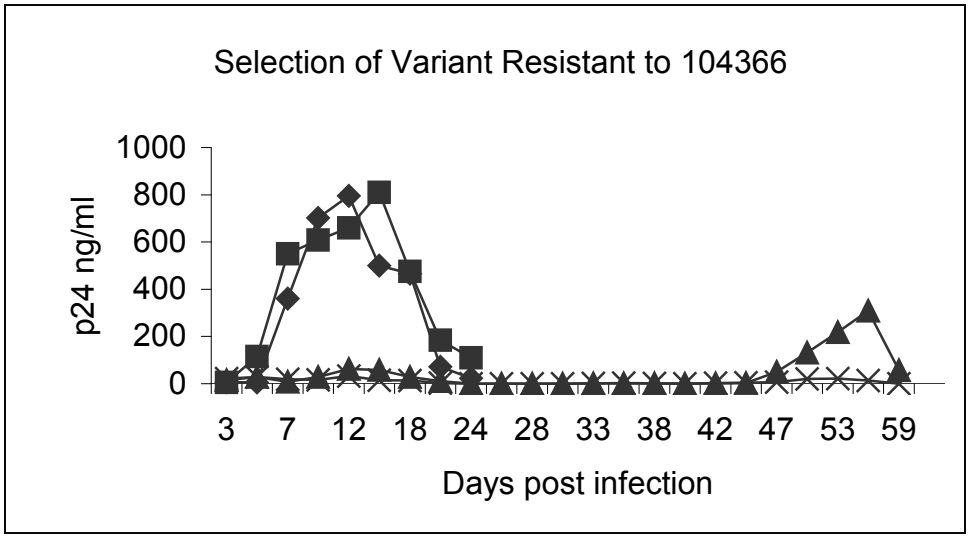


Figure 17. Selection of Variant Resistant to 104366.

G11 cells were infected with NL4-3 nef (-) using polybrene method described in methods. Viral control cultures were grown in RPMI-1640 media supplemented with 10% Fetal Calf Serum and 50μg/ml Gentamycin Sulfate in duplicate without drug (◆ ■), or with either 0.8μM (▲ x) or 8μM of 104366 (data not shown). ELISA was used to measure the p24 released into the medium at each time point. Duplicate cultures shown. 8μM cultures are not shown as they were completely inhibited for the entire time period.

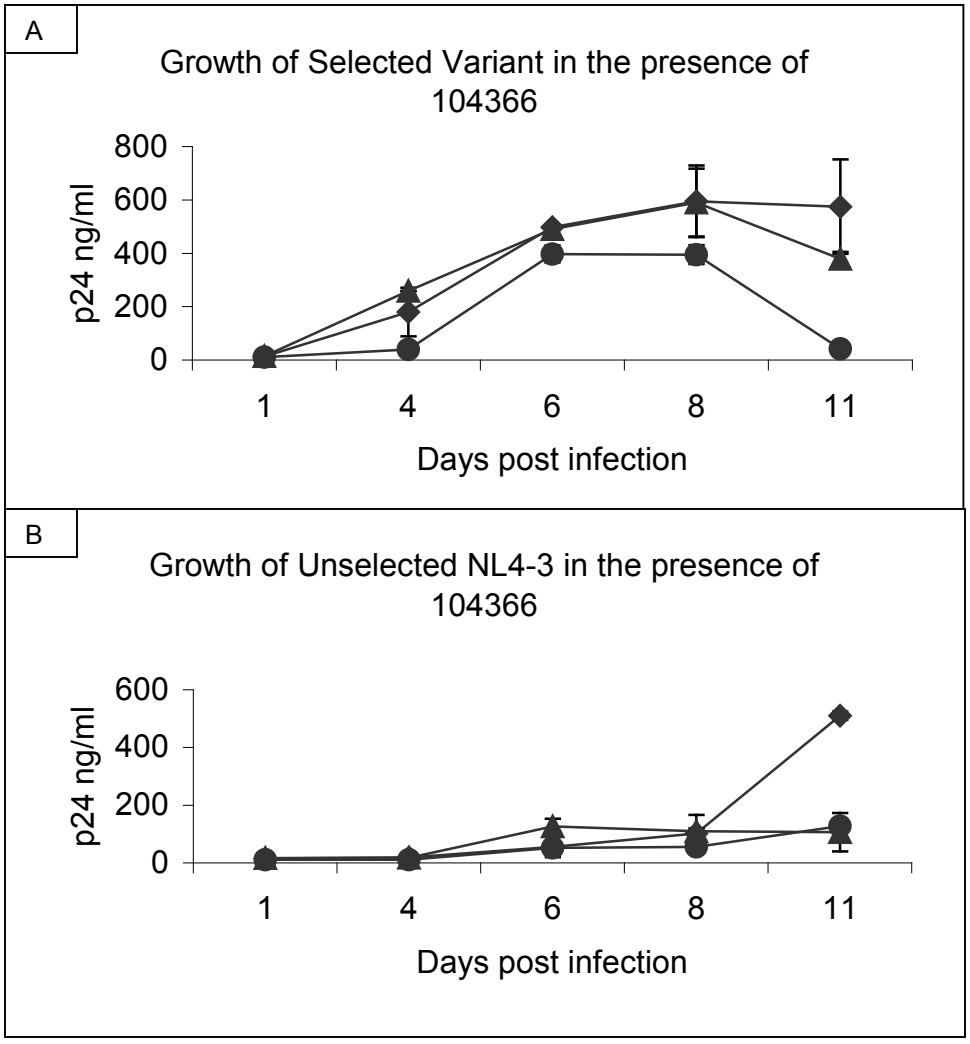


Figure 18. A: Growth of 104366 selected virus in SupT1 cells.

Viral supernatant from Day 53 of 0.8μM culture shown in Figure 15 was used to infect SupT1 cells with no drug (◆), 0.8μM 104366 (▲), or 8μM 104366 (●). B: Infection of SupT1 cells by NL4-3 nef (-). Parent virus was grown in no drug (◆), 0.8μM 104366 (▲), or 8μM 104366 (●). For both A and B ELISA used to measure p24 released into medium at each time point indicated. Curves represent average of duplicate cultures with error bars shown.

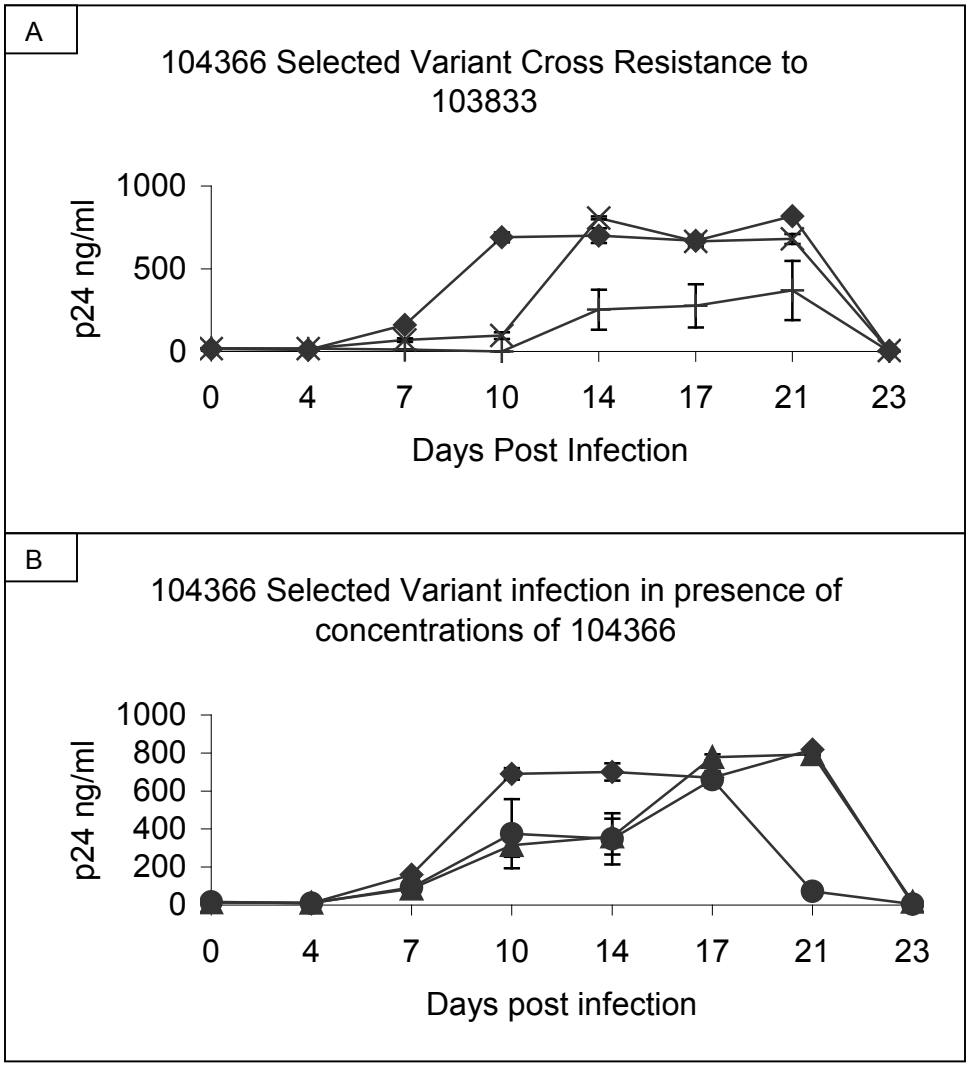


Figure 19. 104366 Selected Variant Cross Resistance to 103833.

A: Day 53 virus was used to infect SupT1 cells in the presence of 103833. Curves represent duplicate samples with error bars shown: no drug (◆), 1μM 103833 (X), 10μM 103833 (+)

B: Growth of 104366 Resistant Variant in SupT1 cells. Curves represent duplicate samples with error bars shown: no drug (◆), 0.8μM 104366 (▲), 8μM 104366 (●)

Selection of resistance to compound 103833

We used a different protocol to isolate a viral variant resistant to 103833. The strategy in this case, was to do serial passages of virus exposed to suboptimal concentrations of drug. To start, SupT1 cells were infected with virus in the presence of either 1μM or 10μM 103833 (Figure 20 panel A). The initial infection produced minimal viral growth in the 1μM culture and virtually no growth at 10μM 103833. Supernatant from the peak of the 1μM culture (day 14) was collected and used to infect SupT1 cells of the next passage in the presence of both drug concentrations (Figure 20 panel B). As before the 1μM culture from this passage also produced viral growth, but this time to a slightly higher level. Again supernatant from the peak of the second passage (day 10) was used to infect SupT1 cells for the third passage (Figure 20 panel C). By the third passage the virus was able to replicate to similar levels at both concentrations of drug tested.

The 103833 resistant virus was next tested for cross resistance to compound 104366. To do this virus from the third passage (day 7) was used to infect SupT1 cells in the presence of 104366. As a control this virus was also cultured in the presence of 103833 to confirm resistance. Figure 21 panel A illustrates the growth of the 103833 selected variant in the presence of 0.8μM or 8μM 104366, demonstrating cross resistance. Figure 21 panel B confirms the resistance of the variant to 103833 at the concentrations tested (1μM and 10μM).

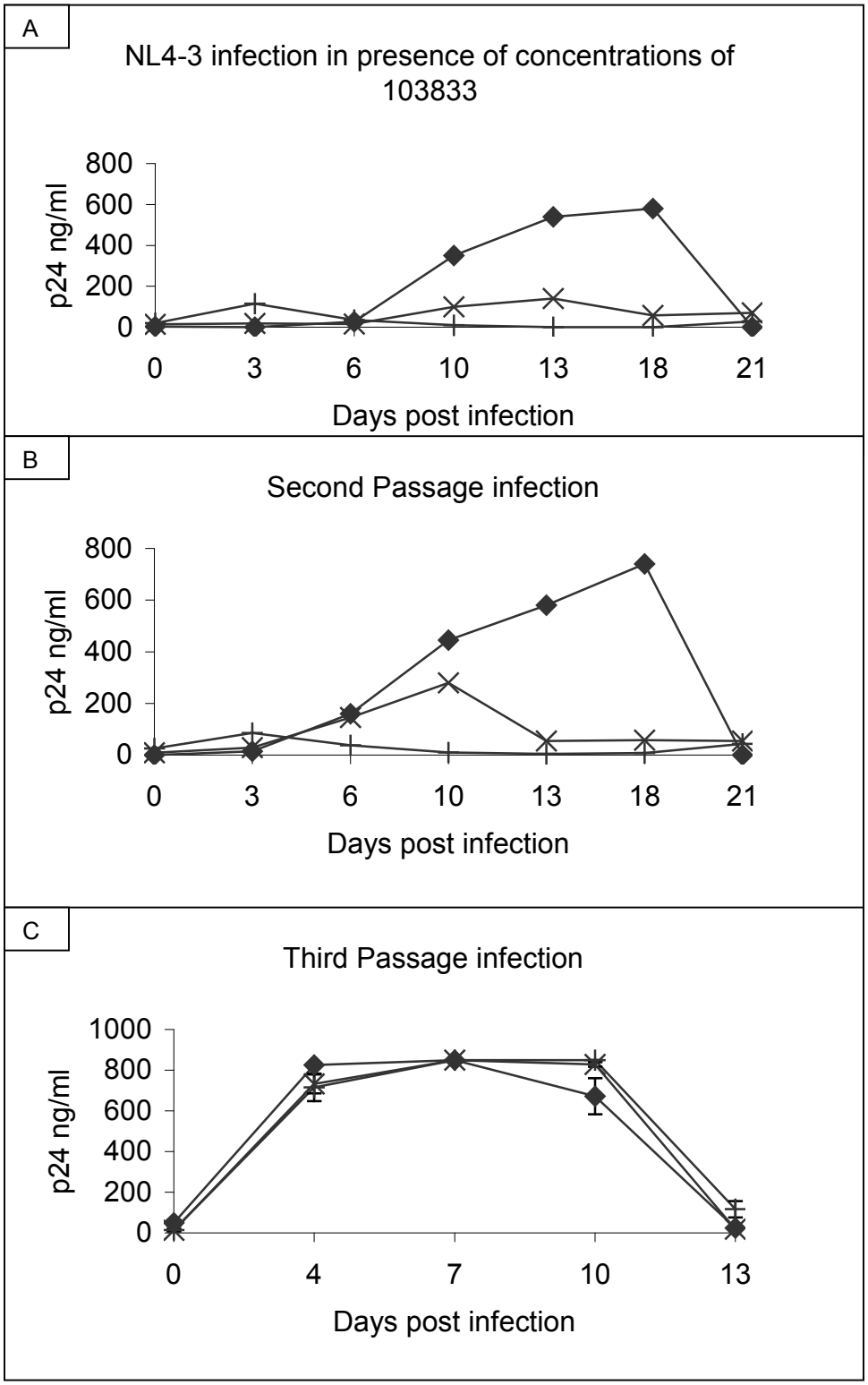

A
NL4-3 infection in presence of concentrations of 103833
p24 ng/ml
800
600
400
200
0
0
3
6
10
13
18
21
Days post infection
B
Second Passage infection
p24 ng/ml
800
600
400
200
0
0
3
6
10
13
18
21
Days post infection
C
Third Passage infection
p24 ng/ml
1000
800
600
400
200
0
0
4
7
10
13
Days post infection

Figure 20. Selection of Variant Resistant to 103833.

NL4-3 nef (-) was used to infect SupT1 cells. Cultures grown in 1μM were serially passaged (used to infect subsequent cultures) for selection of a resistant variant.

A: Growth of NL4-3 in the presence of 103833 (first passage)

no drug (◆), 1μM 103833 (×), 10μM 103833 (+)

B: Growth of 1μM peak from day 14 of first passage (second passage)

no drug (◆), 1μM 103833 (×), 10μM 103833 (+)

C: Growth of 1μM peak from day 10 of second passage (third passage)

Curves represent duplicate cultures with error bars shown

no drug (◆), 1μM 103833 (×), 10μM 103833 (+)

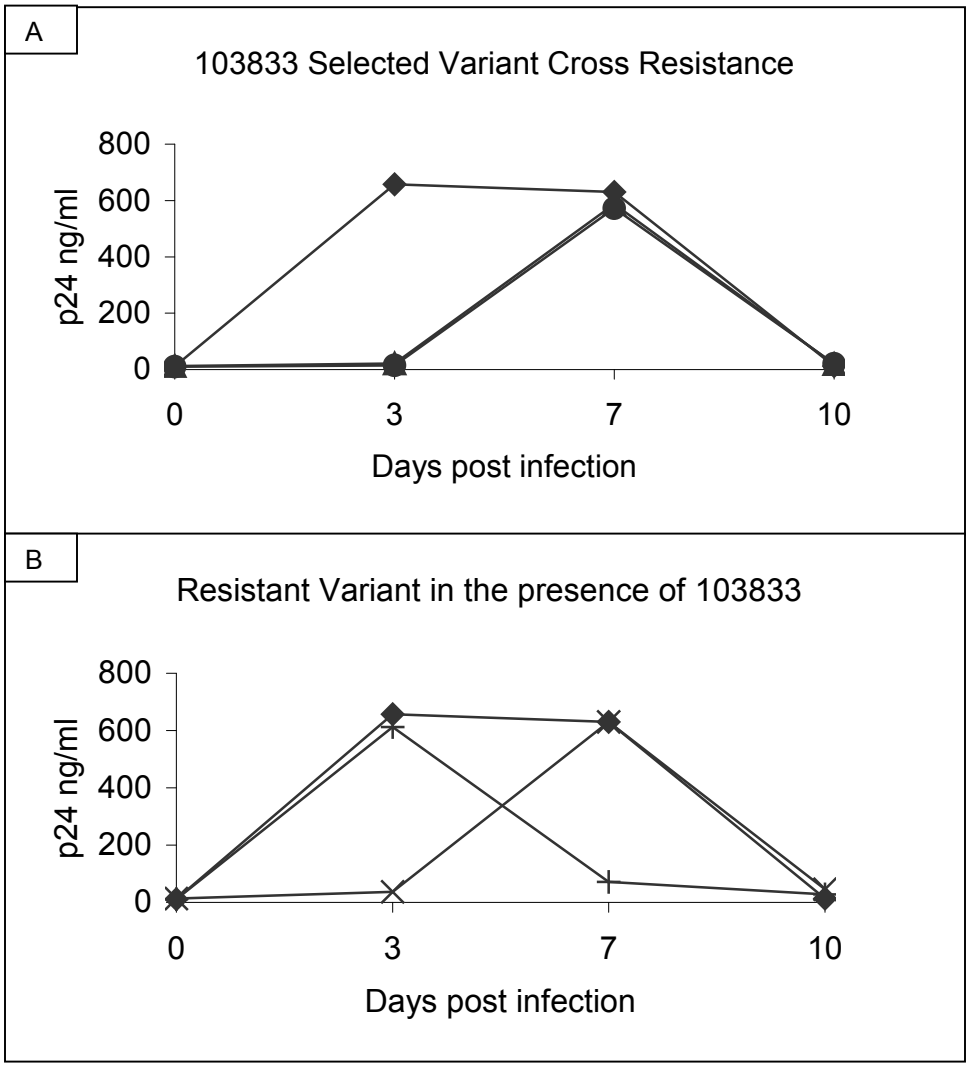


Figure 21. Virus from the third pass (day 7) was used to infect SupT1 cells to test for cross resistance to 104366, and to confirm resistance to 103833. ELISA was used to measure p24 levels at each time point indicated. A: Growth of 103833 selected variant with no drug (◆), 0.8μM 104366 (▲), 8μM 104366 (●). B: Growth of 103833 selected variant with no drug (◆), 1μM 103833 (×), 10μM 103833 (+)

Testing for reversion of phenotype in the absence of selective pressure

Both resistant variants were grown for ten passages in the absence of compounds to test for reversion back to the wild type virus. To do this supernatant from the peak growth of each passage, as determined by p24 ELISA, was used to infect the next passage (data not shown). Each passage peak was also tested in separate cultures in the presence of the compounds, to determine if the viruses remained resistant and cross resistant as previously demonstrated. After ten passages the resistant variants never demonstrated reversion of phenotype. As an example, the data from the virus from the last passage is shown (Figure 22). The data showed that the virus remained resistant to the compounds at the concentrations tested.

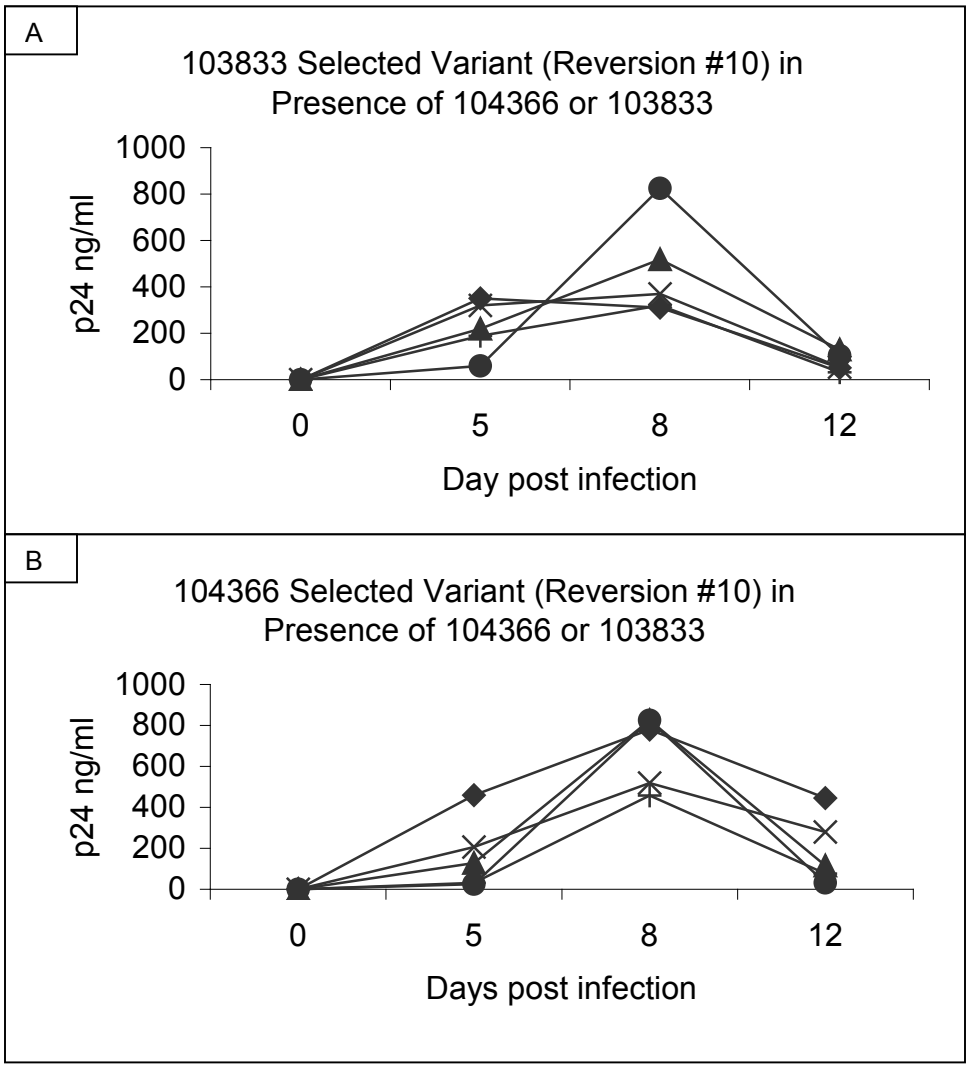


Figure 22. Testing for Reversion of Resistant Variants.

A: After ten serial passages of the 103833 selected variant in the absence of compound, virus was used to infect SupT1 cells with no drug (◆), 0.8μM 104366 (▲), 8μM 104366 (●), 1μM 103833 (×), 10μM 103833 (+). B: After ten serial passages of the 104366 selected variant in the absence of compound, virus was used to infect SupT1 cells with no drug (◆), 0.8μM 104366 (▲), 8μM 104366 (●), 1μM 103833 (×), 10μM 103833 (+)

Genotype of the resistant viruses

In order to identify mutations in the resistant variants, viral RNA was isolated, and two large fragments of interest covering *rev*, *tat*, *vpu*, *env*, and the RRE were amplified into cDNA by RT PCR as described in the methods. DNA Sequencing of the PCR products revealed mutations in both the RRE and *env* for both the 104366 and 103833 resistant variants. Figure 23 diagrams the location of the two overlapping fragments in NL4-3. The fragments include unique restriction enzyme sites (SalI-NheI and NheI-XhoI) used for cloning purposes. Nucleotide locations of the sites are annotated in Figure 23.

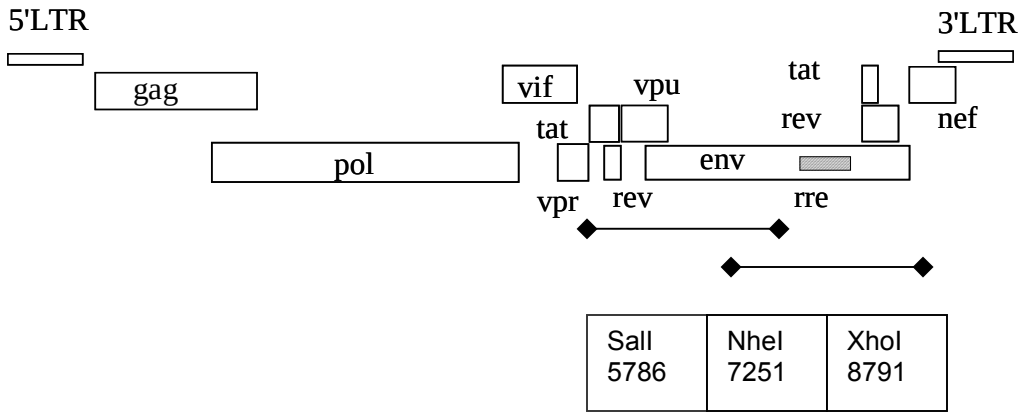


Figure 23. Schematic of NL4-3 genetic areas showing SalI to NheI and NheI to XhoI overlapping PCR fragments used for DNA sequencing.

For the 104366 variant PCR fragments were cloned into Invitrogen TOPO Clone pCR4 vectors, and individual clones were isolated and sequenced. Table 2 illustrates the mutations contained in these analyzed clones. The mutations A7854G and A7936G were of particular interest because of their location in the RRE.

The 103833 resistant variant was not analyzed in the same manner. Instead sequencing was performed on the bulk PCR products, and a single mutation C7836T was identified in the RRE. To study the phenotype of the C7836T mutation, it was created by site mutagenesis of the wild type RRE. This mutation was of great interest because it corresponded as the binding partner of A7854 in the RRE structure. Interestingly the A7854G mutation identified in the 104366 resistant variant, and the C7836T mutation identified in the 103833 resistant variant, both allowed for the formation of another nucleotide binding pair at the base of stem loop IIC in the RRE. These mutations also caused the corresponding amino acid changes, D36G and A30V, in gp41. Figure 24 illustrates these mutations on the NL4-3 RRE structure.

Clones containing mutation	Nucleotide change	Triplet change	Amino acid change	Location NL4-3 nt
1.3, 9.3, 13.10	A→G	GAT→GGT	D→G	RRE 7854
1.3, 5.7, 9.3	A→G	AAA→AAG	K no change	RRE 7936
5.7	A→C	AAC→CAC	N→H	Env gp41 8086
5.7	T→C	ATG→ACG	M→T	Env gp41 8091
9.3	G→A	AAG→AAA	K no change	Env gp41 8179
9.3	C→T	GGC→GGT	G no change	Env gp41 8764

Table 2. Mutations identified in the four 104366 selected variant clones.

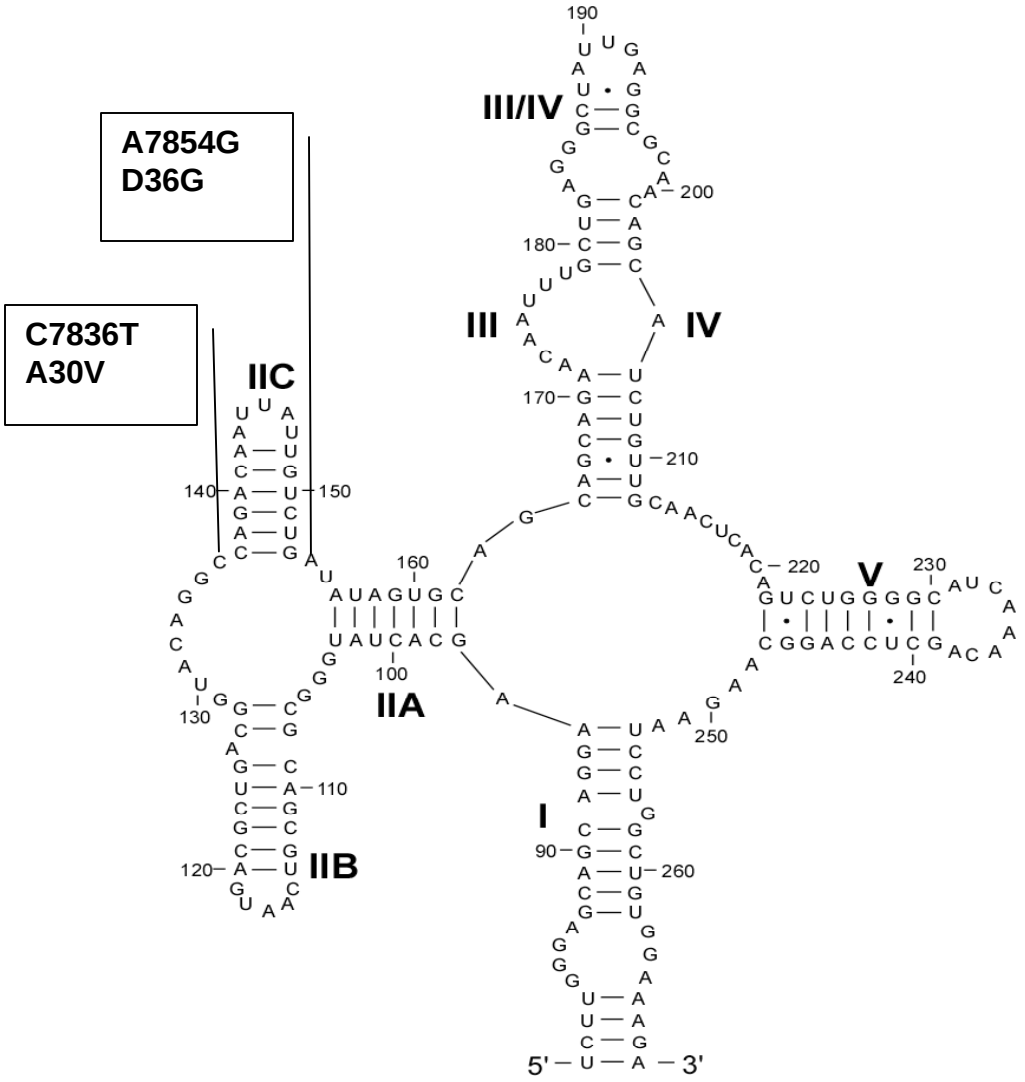


Figure 24. Structure of NL4-3 RRE with Resistance Mutations Identified in 104366 and 103833 Selected Variants.

Both mutations cause formation of additional nucleotide binding pair at the bottom of stem loop IIC, and also change the amino acid sequence of gp41 as annotated. When built back into the wild type NL4-3 backbone, both mutations were shown to confer resistance to both 103833 and 104366.

Fragments containing the two mutations described above, as well as other mutations identified in sequencing the PCR products were built back into the wild type NL4-3 nef (-) proviral background as described in the methods. These proviruses were used to produce virus that was then cultured in SupT1 cells in the presence or absence of compounds. Two viruses out of the five that were tested in this way showed the resistant phenotype. These viruses had the single point mutations described above. The mutations are illustrated in Figure 24 on the NL4-3 RRE. Both viruses were resistant to both compounds at the concentrations tested (Table 3). The other viruses did not grow with normal fitness or were not resistant to the compounds (Table 3). Figure 25 illustrates the viral growth curves of the A7854G and C7826T viruses in the presence of 103833 or 104366. As a control these resistant mutants were grown in tandem with NL4-3, which again demonstrated inhibited by the compounds.

Proviral Clone	Nucleotide changes	Viral Resistance to 103833 and 104366	Viral Growth in SupT1 cells
3536 (1.3 insert)	A7854G A7936G	Partial Sensitivity	Normal
3537 (5.7 insert)	A7936G A8086C T8091C	Sensitive	Normal
3538 (9.3 insert)	A7854G A7936G G8179A C8764T	Sensitive	Poor Growth
3539 (13.10 insert)	A7854G	**Resistant**	Normal
3543 (site mutagenesis)	C7836T	**Resistant**	Normal

Table 3. Mutations Build Back into NL4-3.

The table shows the proviral constructs used to produce virus to test which mutations conferred resistance in the selected variants. The corresponding viral resistance and growth patterns are listed.

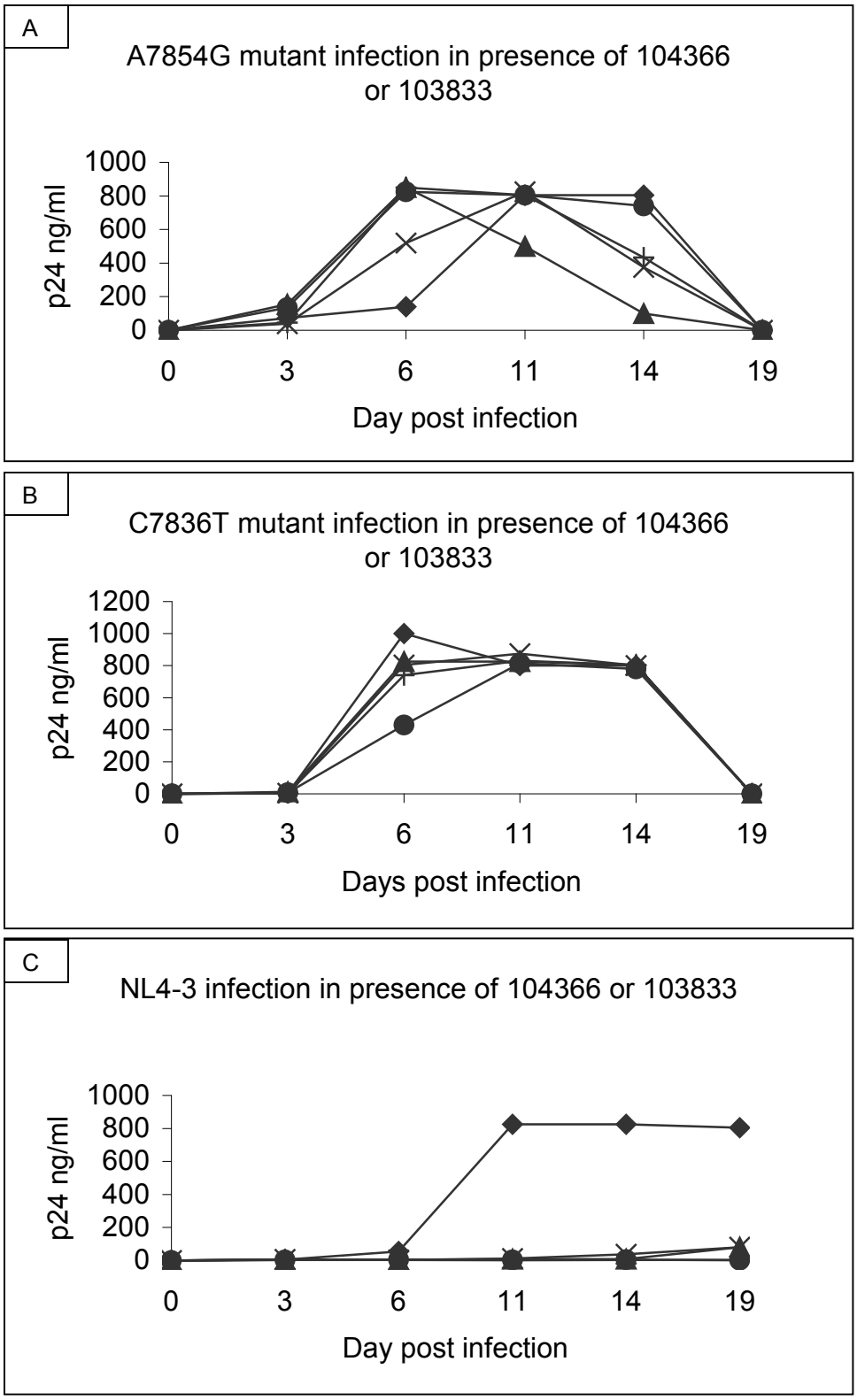

A
A7854G mutant infection in presence of 104366 or 103833
p24 ng/ml
1000
800
600
400
200
0
0
3
6
11
14
19
Day post infection
B
C7836T mutant infection in presence of 104366 or 103833
p24 ng/ml
1200
1000
800
600
400
200
0
0
3
6
11
14
19
Days post infection
C
NL4-3 infection in presence of 104366 or 103833
p24 ng/ml
1000
800
600
400
200
0
0
3
6
11
14
19
Day post infection

Figure 25. Mutations Built Back into Wild Type Background Compared to NL4-3.

Mutations were subcloned into the wild type background. The resultant viruses were tested in SupT1 infections to confirm the relationship to the resistance phenotype. ELISA used to measure p24 levels in the supernatant at each time point indicated.

A: Growth of A7854G in NL4-3 background with no drug (◆), 0.8μM 104366 (▲), 8μM 104366 (●), 1μM 103833 (×), 10μM 103833 (+)

B: Growth of C7836T in NL4-3 background with no drug (◆), 0.8μM 104366 (▲), 8μM 104366 (●), 1μM 103833 (×), 10μM 103833 (+)

C: Growth of NL4-3 nef (-) wild type parent virus with no drug (◆), 0.8μM 104366 (▲), 8μM 104366 (●), 1μM 103833 (×), 10μM 103833 (+)

Response to Rev-RRE inhibitors in Transient Transfections

In order to determine if the resistance mutations in the RRE directly conferred resistance in first round synthesis, we performed transient transfection experiments using NL4-3 proviruses that contained mutant RREs. NL4-3 with the wild type RRE was used as a control. The transient transfection assay measures viral production directly from the proviral DNA. Because gene expression comes directly from the transfected proviral clone, the envelope protein is not a factor in viral production. Thus, unlike in a viral infection, the changes in gp41 amino acid sequence conferred by the resistance mutations would not have an effect on the results of this assay.

To perform this experiment, 293T cells were transfected with proviral clones carrying either the wild type RRE, the RRE with the A7854G mutation, or the RRE with the C7836T mutation. Samples were taken at 72 hours for p24 analysis to measure the amount of virus produced. Transfection of 293T cells by either wild type or mutant RREs showed that the compounds act in a manner similar to what was observed in culture. The compounds failed to inhibit proviruses containing the mutant RREs at the concentrations tested. This result suggests the structural changes in the RRE conferred by the mutations cause the resistant phenotype, not the changes in the envelope protein sequence. Figure 26 shows transfection experiments testing both compounds (panel A) or the 103833 compound at two separate concentrations (panel B).

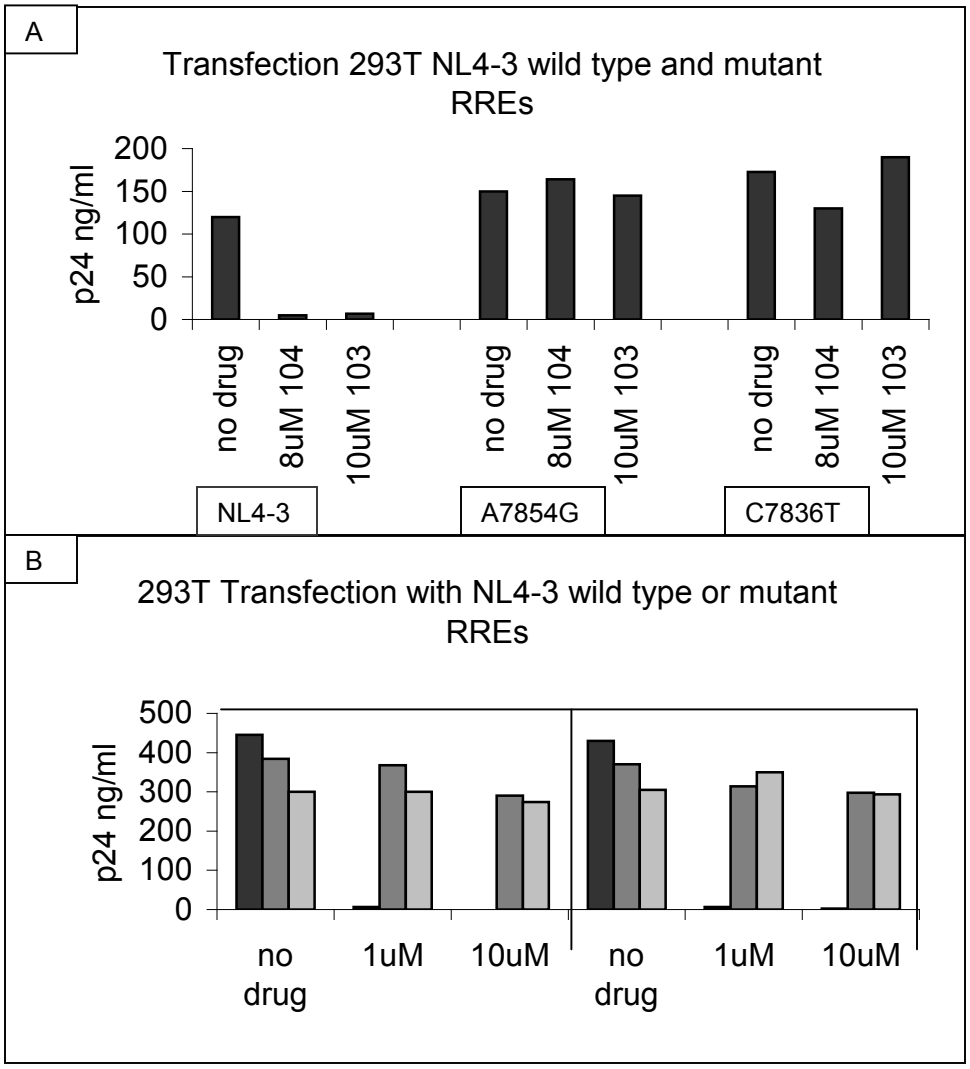


Figure 26. 293T Transfections in the presence of 104366 or 103833.

A: Bars from left to right: Wild type RRE with no drug, 8μM 104366, or 10μM 103833; A7854G RRE with no drug, 8μM 104366, or 10μM 103833; C7836T RRE with no drug, 8μM 104366, or 10μM 103833.

B: Wild type RRE (black), A7854G mutant RRE (dark gray), C7836T mutant RRE (light gray) with no drug, 1μM 103833, or 10μM 103833. Duplicate transfections shown separated by lines.

We next utilized site mutagenesis to disrupt the stabilization of stem loop IIC, created by these resistance mutations, while maintaining the amino acid changes in gp41 (A30V and D36G). To do this we disrupted the base pair that normally resides above or the potential base pair below the extra base pair formed by the resistance mutations (C7837A disrupts C-G base pair above, U7855A mutation disrupts the potential G-U base pair below). Since the new mutations alter the third nucleotide in the gp41 codons, they do not change amino acids 30 or 36. Figure 27 illustrates the original RRE structure and sequence, the resistance mutations A7854G and C7836T, and the disruption mutations C7837A and U7855A.

The mutagenized RREs were built back into the wild type background, and the mutant viruses were grown in SupT1 cells to test for growth in the presence of the compounds. Virus with disruption of stem loop IIC either above, or both above and below the resistance mutations, no longer was resistant to either compound. Figure 27 illustrates the RRE structure and sequence with these mutations. Figure 28 shows the growth curves of the A7854G disruption mutants. The compounds inhibited these viruses similarly to the NL4-3 wild type parent (data not shown). Figure 29 shows the growth curves of the C7836T disruption mutants with similar results to Figure 28. Overall these results show that the amino acid changes in gp41 were not responsible for the resistant phenotype. As previously concluded the structural changes in the RRE stem loop IIC allowed for resistance.

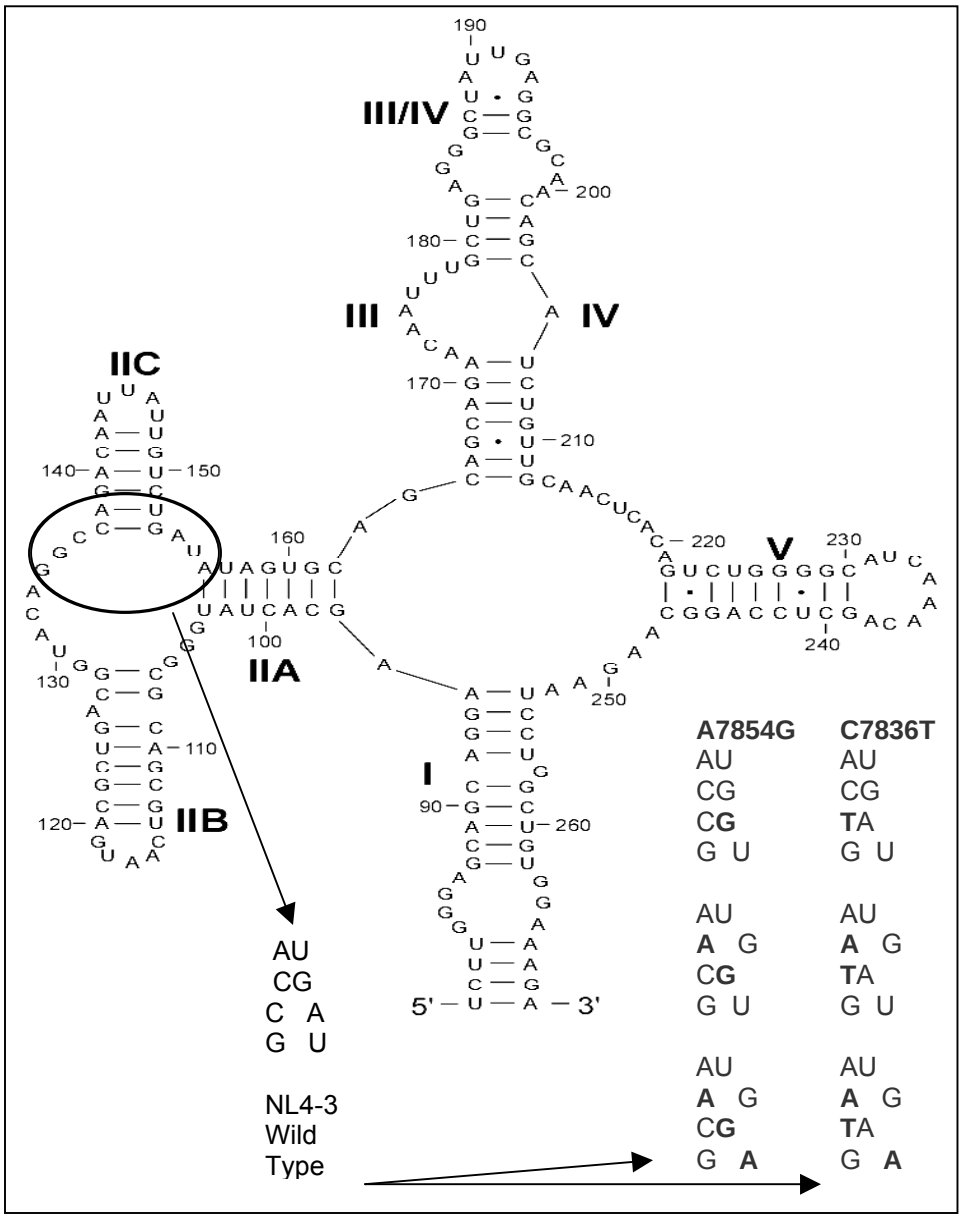


Figure 27. Site Mutagenesis of Resistant RREs selected by 104366 (A7854G) or 103833 (C7836T) in 3rd position of codon for amino acids 30 and 36. Structural disruptions above, or above and below, binding pair of resistance mutations are shown (original mutations and disruptions in bold).

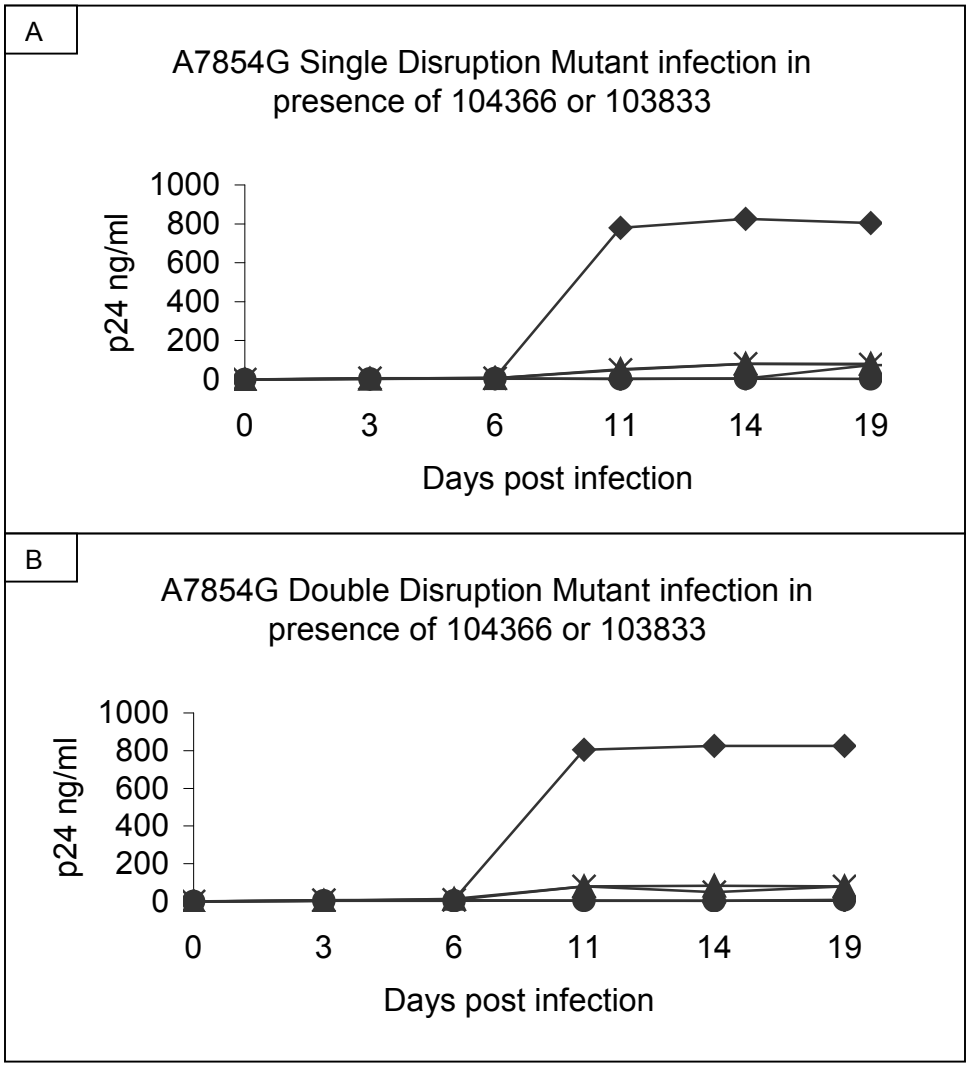


Figure 28. Site directed Mutagenesis of the A7854G mutant.

Mutagenized viruses were tested in SupT1 infections for p24 production (measured by ELISA) in the presence of 103833 or 104366. A: Growth of A7854G and C7837A virus with no drug (◆), 0.8μM 104366 (▲), 8μM 104366 (●), 1μM 103833 (×), 10μM 103833 (+)

B: Growth of A7854G, C7837A, and U7855A virus with no drug (◆), 0.8μM 104366 (▲), 8μM 104366 (●), 1μM 103833 (×), 10μM 103833 (+)

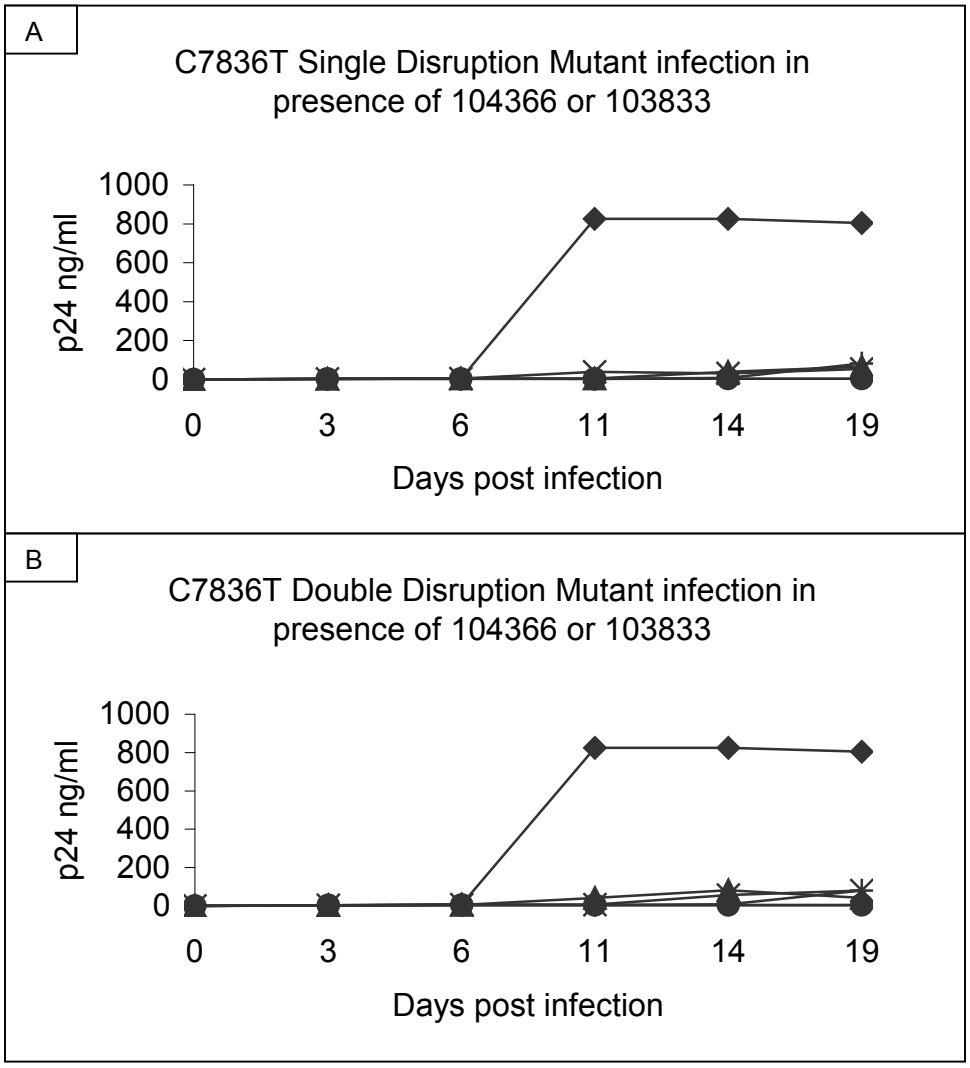


Figure 29. Site directed mutagenesis of the C7836T mutant.

Mutagenized viruses were tested in SupT1 infections for p24 production (measured by ELISA) in the presence of 103833 or 104366. A: Growth of C7836T and C7837A virus with no drug (◆), 0.8μM 104366 (▲), 8μM 104366 (●), 1μM 103833 (×), 10μM 103833 (+)

B: Growth of C7836T, C7837A, and U7855A virus with no drug (◆), 0.8μM 104366 (▲), 8μM 104366 (●), 1μM 103833 (×), 10μM 103833 (+)

Rev Titration Assays Testing Difference in RRE Function

Since the RRE was shown to be the determinant for resistance, we next examined whether the wild type and mutant RREs differed in their responses to the Rev protein. As described in the methods we performed Rev titrations with GagPol constructs, under the control of the pCMV promoter, containing the wild type or mutant RREs. These constructs lack all of the HIV genes except GagPol. The GagPol RRE constructs with the A7854G and C7836T mutations responded to lower Rev levels than the wild type NL4-3 RRE (Figure 30), with the A7854G mutation giving higher p24 values than the C7836T mutation. The shape of the dose response curves are also different between the mutant and wild type RREs in that both mutants seem to reach a plateau quickly. Even though not completely understood, the ability of the mutant RREs to respond to the lower levels of Rev helps explain this as Rev-RRE saturation. As another control we also tested a GagPol construct with a RevM10 resistant NL4-3 RRE, with mutations in the central loop and stem loop V (74). This construct behaved similar to the wild type NL4-3 RRE. Neither NL4-3 nor the RevM10 GagPol constructs resulted in a plateau at the Rev concentrations tested indicating the RREs were not saturated with Rev. So only the changes in stem loop IIC caused improved Rev-RRE function.

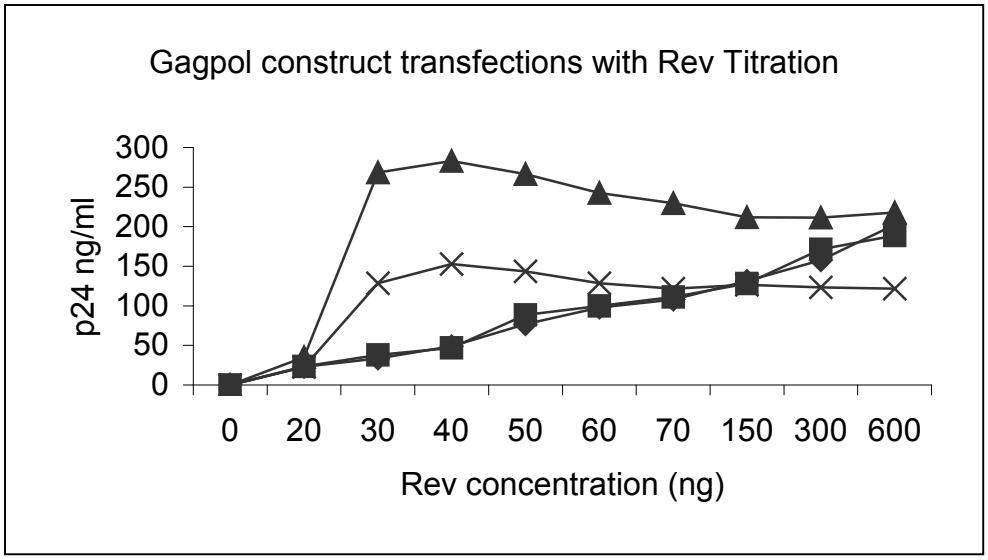


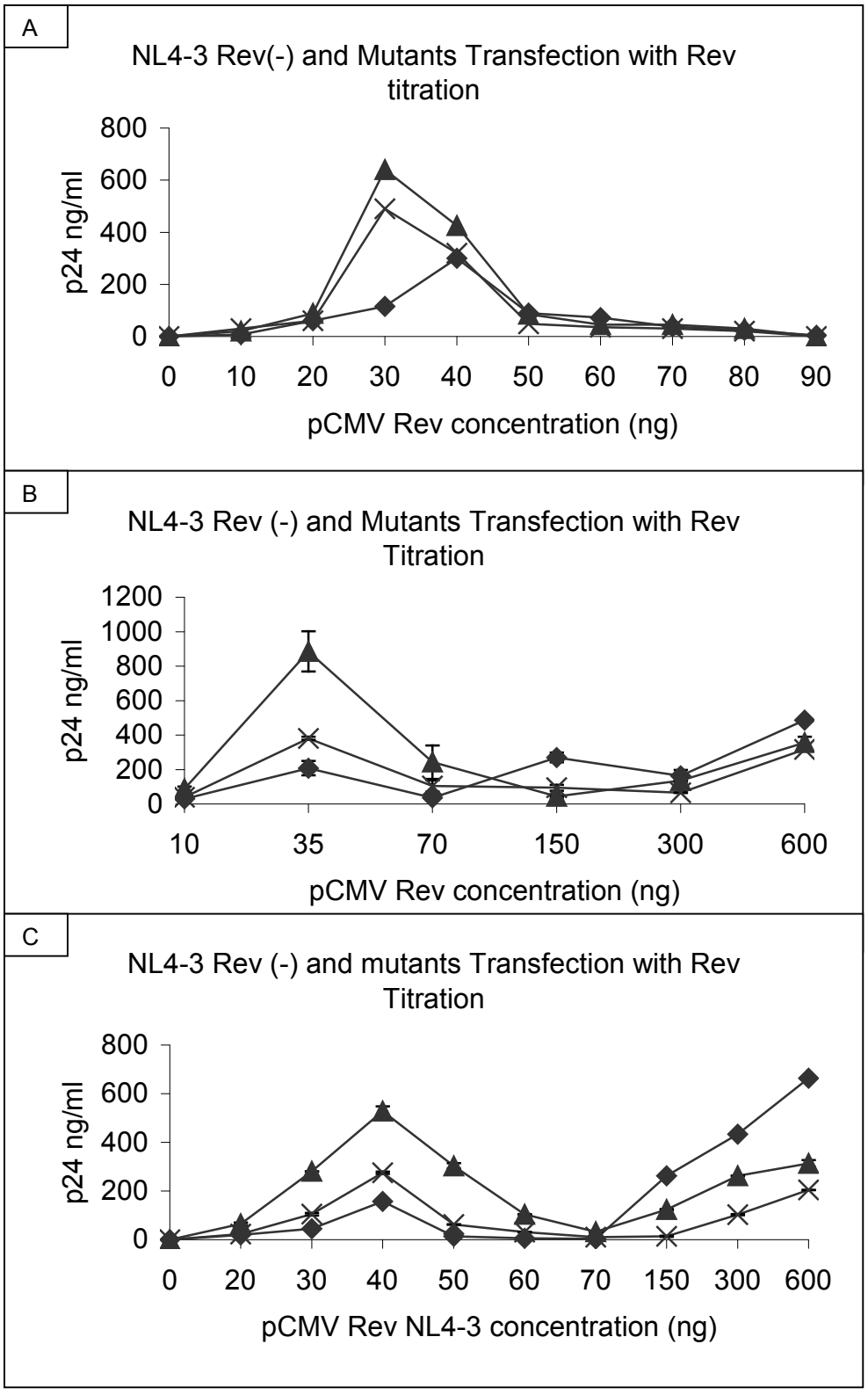
Figure 30. GagPol Rev Titration.

pCMVGagPol constructs with either NL4-3 or Mutant RREs transfection of 293T cells with pCMVRev titration from 0-600ng.

Curves represent average of two separate transfections:

GagPol construct with NL4-3 RRE (◆), A7854G RRE (▲), C7836T RRE (×), RevM10 mutant RRE (■). ELISA used to measure p24 levels in the transfection supernatant.

We then performed transient transfections of 293T cells using Rev (-) proviruses, containing either wild type or mutant RREs, with increasing concentrations of Rev supplied in trans. We evaluated the differences in viral production at the various Rev levels between the RREs and observed similar results to the GagPol assay (Figure 31). Curiously we noted concentrations of Rev that had a negative effect on viral production. This biphasic response was demonstrated repeatedly in several titrations. This effect was not observed in the GagPol constructs that lacked the viral accessory proteins. Further evaluation of this effect was done at a later time as will be shown in Figure 36.


A
NL4-3 Rev(-) and Mutants Transfection with Rev titration
p24 ng/ml
800
600
400
200
0
0 10 20 30 40 50 60 70 80 90
pCMV Rev concentration (ng)
B
NL4-3 Rev (-) and Mutants Transfection with Rev Titration
p24 ng/ml
1200
1000
800
600
400
200
0
10 35 70 150 300 600
pCMV Rev concentration (ng)
C
NL4-3 Rev (-) and mutants Transfection with Rev Titration
p24 ng/ml
800
600
400
200
0
0 20 30 40 50 60 70 150 300 600
pCMV Rev NL4-3 concentration (ng)

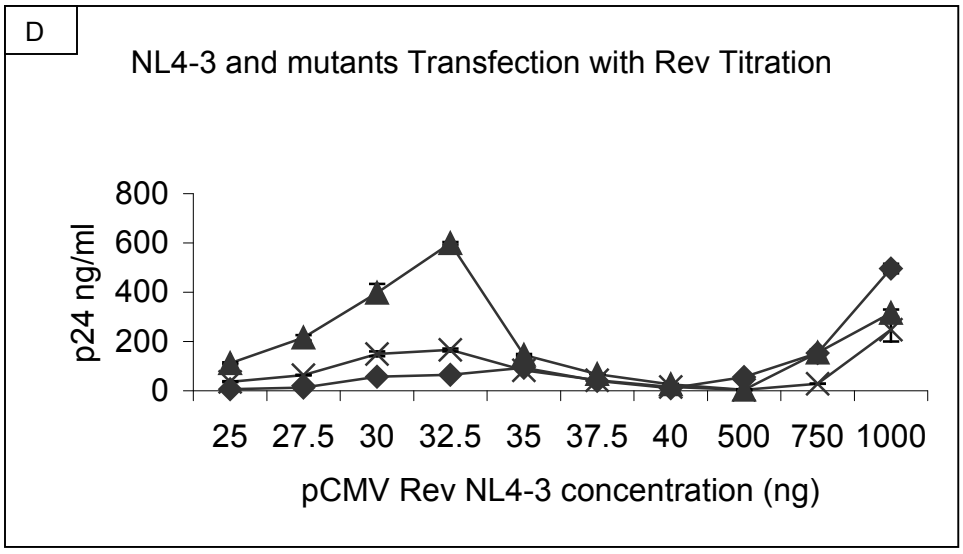


Figure 31. Mutant Versus Wild Type RREs in NL4-3 Provirus Rev Titrations.

A: 5μg pNL4-3 or Mutant RRE Rev (-) provirus transfection of 293T cells with pCMVRev titration from 0-90ng.

B: 5μg pNL4-3 or Mutant RRE Rev (-) provirus transfection with pCMVRev titration of 10, 35, 70, 150, 300, and 600ng Rev.

C: Expanded pCMVRev titration from 0-70ng, and also 150, 300, 600ng pCMVRev.

D: Further expanded pCMVRev titration from 25-40ng Rev, and also 500, 750, 1000ng pCMVRev.

For all panels each curve represents duplicate samples with error bars shown: NL4-3 (◆), A7854G RRE (▲), C7836T RRE (×). ELISA used to measure p24 in the transfection supernatant.

Study of HIV-1 Clade B virus R73

The RREs from NL4-3 demonstrated that A7854G confers resistance to both 103833 and 104366 by structurally changing the RRE to use lower functional Rev levels. Other RREs that have been tested by SRI and us also contained this change without demonstrating the resistance phenotype under the conditions tested. It is of note that these RREs also contained other changes that could also influence the resistance phenotype. Overall the work done so far was in the context of NL4-3. We were thus interested in studying a virus with a different genetic background. We acquired a HXB2 derivative virus named R73 that lacks Vpu, Vpr, and Nef.

We tested the R73 virus in cell culture for sensitivity to 103833 and 104366. In addition to lacking several proteins, R73 contains an RRE sequence that differs slightly from NL4-3, with a G instead of A at positions 7854 and 7936. Since this virus contained a G at position 7854, which was a resistance mutation in the context of NL4-3, we thought it might demonstrate resistance to the Rev-RRE inhibitors. Thus R73 was cultured in SupT1 cells in the presence of 103833 and 104366. Figure 32 shows that the compounds had some effect on R73 replication, causing delayed growth in a dose dependent manner. Overall R73 did not show sensitivity to the extent of the wild type NL4-3 or the full resistance of the Rev-RRE inhibitor selected variants. We were interested in examining this further by trying to isolate a variant of R73 that was fully resistant.

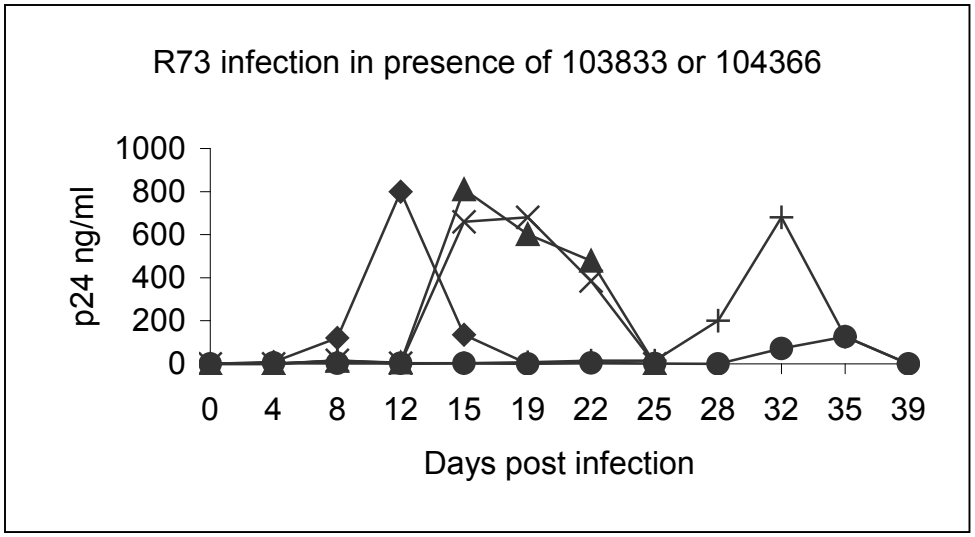


Figure 32. R73 infection of SupT1 cells in the presence of 103833 or 104366 at a viral load of 50ng p24 equivalents. R73 cultures produced virus in a dose dependent manner with respect to both compounds. Curves represent single cultures: no drug (◆), 0.8μM 104366 (▲), 8μM 104366 (●), 1μM 103833 (×), 10μM 103833 (+). ELISA used to measure p24 at each time point shown.

We performed long term selection of R73 in the presence of 103833 at the viral input of the previous cultures. As before, the R73 wild type virus was able to grow in the presence of compounds with only a slight lag. The virus from the peak of growth, was cultured again in SupT1 cells, and did not demonstrate full resistance to the compounds, but grew in a manner identical to the wild type R73 (data not shown). We thus repeated the R73 selection with a viral input five fold lower to allow the compounds to be more effective in controlling viral growth (Figure 33 panel A). The lower viral input shifted the peak of replication of R73 in the absence of drug from 12 to 17 days. This time we were able to recover virus at Day 31 that behaved like resistant variants upon re-culturing. Figure 33 Panel B shows the growth of the resistant variant compared to the R73 parent in the presence of 103833.

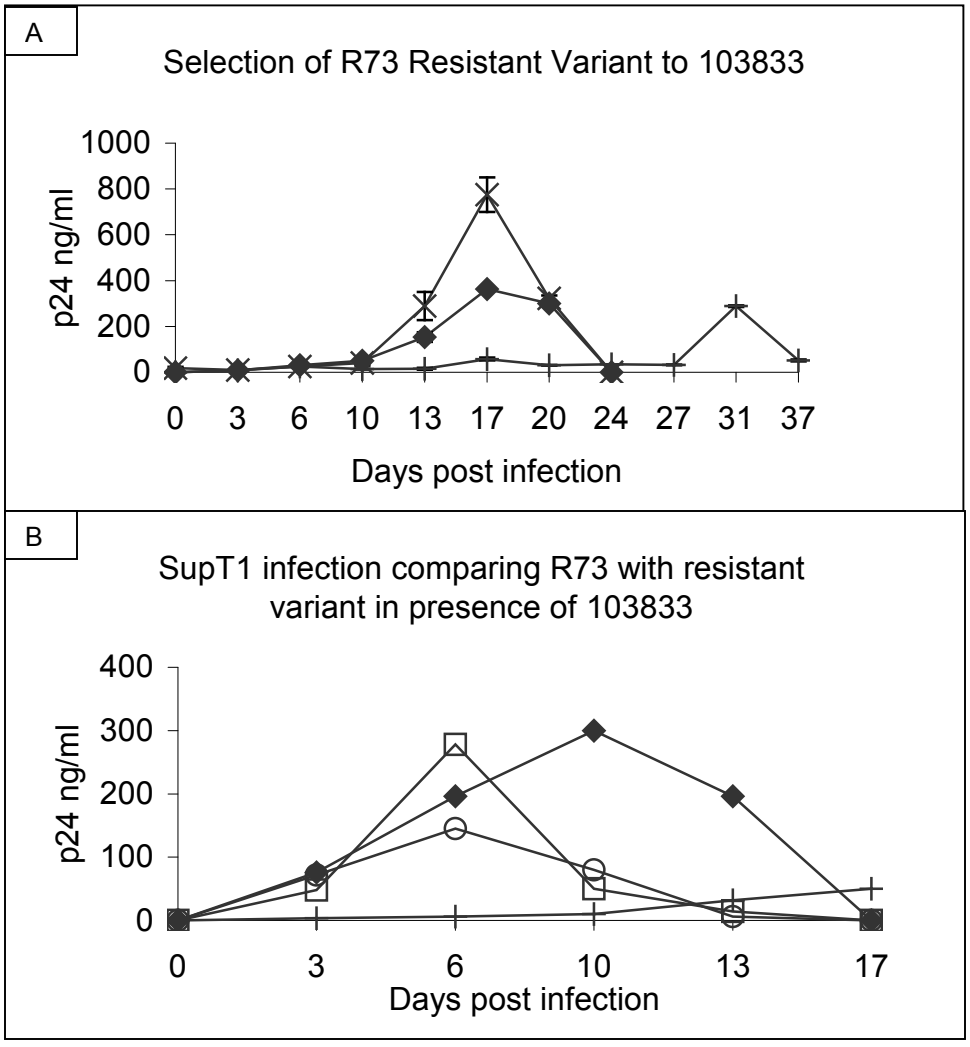


Figure 33. Selection of R73 Variant Resistant to 103833.

A: R73 at a viral load of 10ng p24 equivalents was used to infect SupT1 cells in the presence of 103833. Curves represent duplicates with error bars shown: no drug (◆), 1μM 103833 (×), 10μM 103833 (+) B: Comparison growth curves of R73 parent virus and Day 31 Resistant Variant in SupT1 cells. Curves represent single cultures: R73 no drug (◆), R73 10μM 103833 (+), Day 31 no drug (□), Day 31 10μM 103833 (O). ELISA used to measure p24 values at each point.

In order to identify mutations in the resistant R73 variant, viral RNA was isolated, and two large fragments of interest covering *rev*, *tat*, *env*, and the RRE were amplified into cDNA by RT PCR as described in the methods. Figure 34 demonstrates the areas in R73 analyzed and unique restriction enzyme sites. DNA Sequencing of the PCR products revealed mutations in only gp120, not *rev* or the RRE (Table 4).

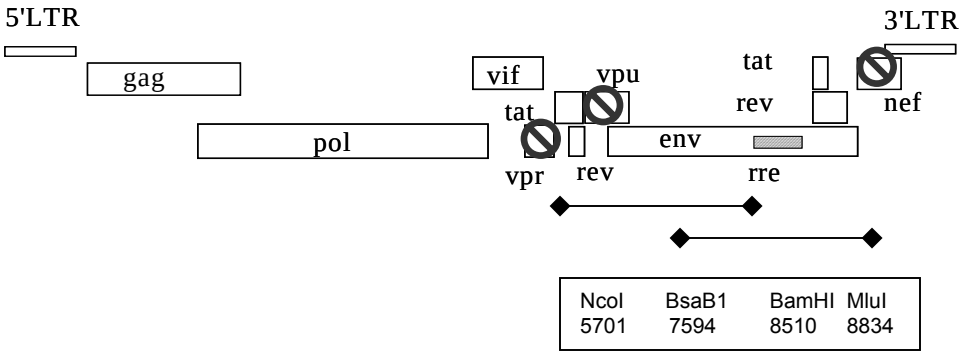


Figure 34. Schematic of R73 genetic locations showing overlapping PCR fragments used in DNA sequencing.

Culture Fig. 33	VIRUS	SELECTION	ORIGINAL RRE	RRE CHANGES	OTHER CHANGES
Panel A +	R73 Day 31	10uM 103833	7854G, stem loop IIC 7936G, stem loop V	NONE	A6402C GCA to GCC A48- gp120 C1, A7295C GAT to GCT D346A gp 120 C3 in HLA epitope

Table 4. Mutations in gp120 from R73 103833 Resistant Variant

We built back the gp120 mutations into the R73 backbone using Site Mutagenesis by Overlap Extension (SOE) PCR and Molecular Cloning as described in the methods. Upon growth of these viruses, only the mutant with both A6402C and A7295C was able to grow in the presence of 10μM 103833. Each single mutation alone did not demonstrate this resistance pattern. Figure 35 panel A shows the growth of the R73 Double Mutant compared to the R73 parent in the presence of 103833. Figure 35 panel B shows the growth of the R73 single mutants A6402C or A7295C in the presence of 103833.

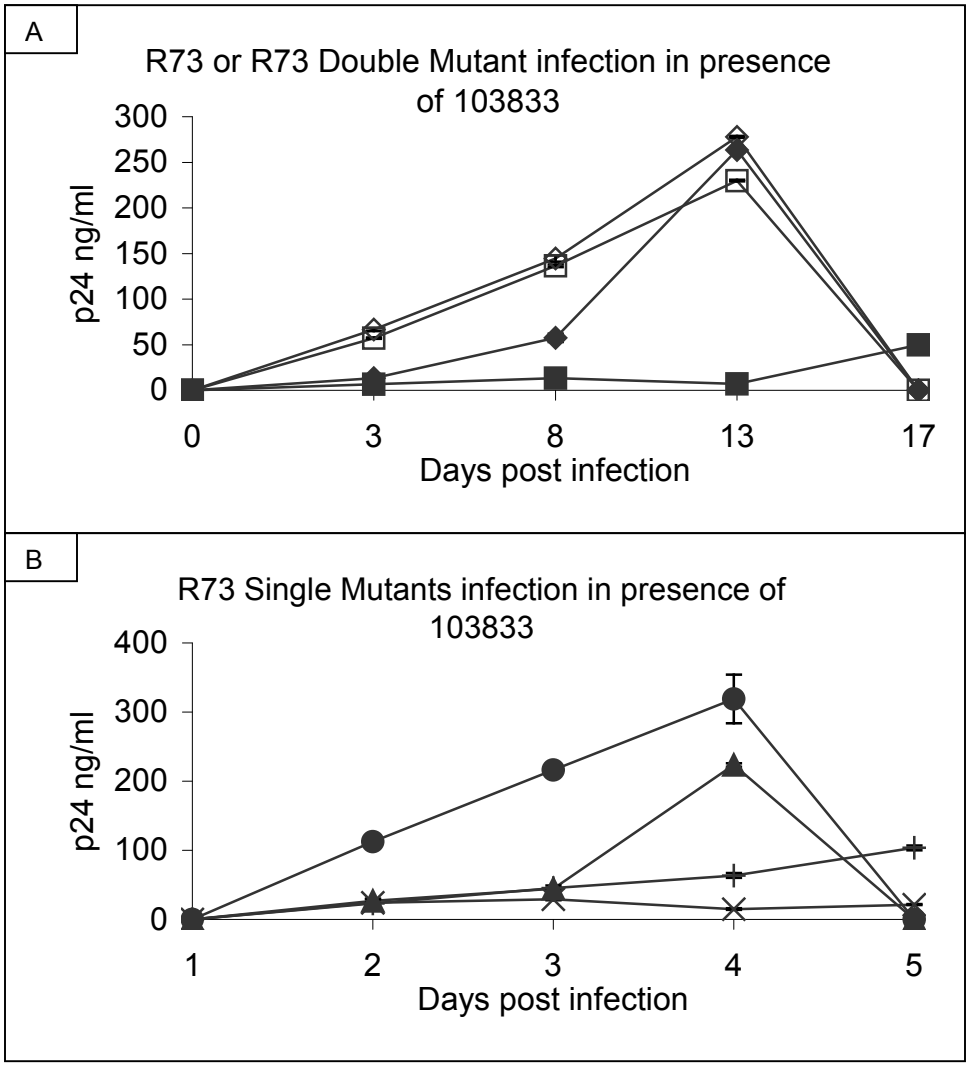

A
R73 or R73 Double Mutant infection in presence of 103833
p24 ng/ml
300
250
200
150
100
50
0
0
3
8
13
17
Days post infection
B
R73 Single Mutants infection in presence of 103833
p24 ng/ml
400
300
200
100
0
1
2
3
4
5
Days post infection

Figure 35. Mutations Built Back into R73 Wild Type Back Bone.

R73 virus and R73 containing mutations identified in resistant variant were used to infect SupT1 cells in the presence of 103833. ELISA used to measure p24 from the culture supernatant at each time point.

A: Double Mutant (A6402C and A7295C) compared to R73 parent virus in the presence of 103833. Curves represent duplicate cultures with error bars shown: R73 no drug (◆), R73 10μM 103833 (■), Double mutant no drug (◇), Double mutant 10μM 103833 (□)

B: Single Mutants (A6402C or A7295C) grown in SupT1 cells in the presence of 103833. Curves represent duplicate cultures with error bars shown: A7295C mutant no drug (▲), A7295C mutant 10μM 103833 (×), A6402C mutant no drug (●), A6402C mutant 10μM 103833 (+)

R73 was used in Rev titration assays to examine the previously observed biphasic response demonstrated by NL4-3. To do this we tested the wild type NL4-3 Rev (-) provirus compared to the Rev (+) NL4-3 and R73 proviruses in transient transfections of 293T cells with pCMVRev supplied in trans. Both Rev (+) and Rev (-) NL4-3 proviruses demonstrated the same negative effect in a similar region of the Rev titration (Figure 36 Panel A and B). The NL4-3 Rev (-) provirus demonstrated the effect earlier in the titration than the Rev (+) provirus. We attributed this to the differences in endogenous Rev. The R73 provirus however was able to produce similar levels of virus despite the Rev titration. This virus contained its own endogenous Rev, so we also created a R73 Rev (-) provirus with the same change in the *rev* exon as the NL4-3 Rev (-) construct. We used these proviruses (R73 Rev (-) and NL4-3 Rev (-)) in the previously described Rev titration assay and again demonstrating the negative effect only in the NL4-3 titration (Figure 36 Panel C). R73 differs from NL4-3 in the expression of accessory proteins. R73 is negative for Vpu, Vpr, and Nef. The GagPol constructs also were negative for Vpu, Vpr, and Nef. We concluded that expression of one of these accessory proteins could be causing this effect in the Rev titration for NL4-3. Also the R73 Rev (-) provirus seemed to peak at a lower Rev level and plateau similar to the mutant RREs. Interestingly the R73 RRE sequence contains the G change like the A7854G mutant in stem loop IIC, and an additional change in stem loop V.

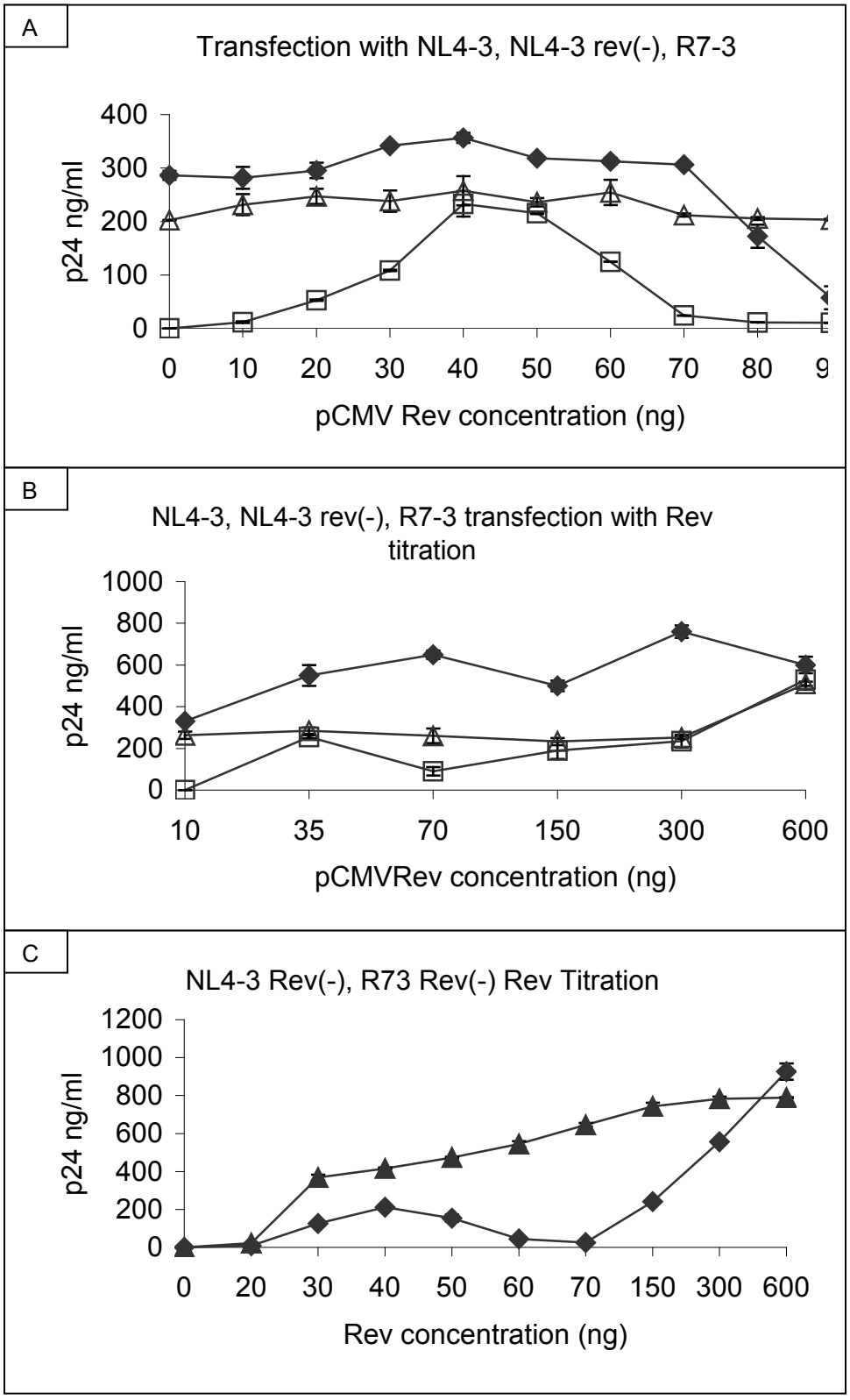

A
Transfection with NL4-3, NL4-3 rev(-), R7-3
p24 ng/ml
400
300
200
100
0
0 10 20 30 40 50 60 70 80
pCMV Rev concentration (ng)
B
NL4-3, NL4-3 rev(-), R7-3 transfection with Rev titration
p24 ng/ml
1000
800
600
400
200
0
10 35 70 150 300 600
pCMVRev concentration (ng)
C
NL4-3 Rev(-), R73 Rev(-) Rev Titration
p24 ng/ml
1200
1000
800
600
400
200
0
0 20 30 40 50 60 70 150 300 600
Rev concentration (ng)

Figure 36. NL4-3 Versus R73 Proviruses Rev Titration.

A: 5μg pNL4-3, pNL4-3 Rev (-), or R7-3 transfection of 293T cells with pCMVRev titration 0-90ng.

Curves represent duplicate samples with error bars shown:

NL4-3 (◆), R7-3 (△), NL4-3 Rev (-) (□)

B: Expanded titration of 10, 35, 70, 150, 300, and 600ng pCMVRev with 5μg pNL4-3, pNL4-3 Rev (-), or R7-3.

Curves represent duplicate samples with error bars shown:

NL4-3 (◆), R7-3 (△), NL4-3 Rev (-) (□)

C: 5μg pNL4-3 Rev (-) or R73 Rev (-) transfection with expanded pCMV Rev titration of 0-70ng, and 150, 300, 600ng.

Curves represent duplicate samples with error bars shown:

NL4-3 Rev (-) (◆), R7-3 Rev (-) (▲)

ELISA used to measure p24 from transfection supernatants.

Infectivity of NL4-3 produced by differing concentrations of Rev

From the transfection data we concluded the mutant and wild type RREs were able to perform with differing functional Rev levels. In order to further understand how Rev levels affected the viral progeny, we investigated whether virus produced in differing levels of Rev would have differences in infectivity. To do this we tested serially diluted NL4-3 (Rev (+) and (-) that was produced with either 35ng or 600ng of trans Rev) in GFP reporter cell lines. We were unable to determine differences in infectivity, the TCID50 of the viruses appeared unchanged.

Interestingly the virus with endogenous Rev, produced with additional 600ng Rev, did show a negative effect on infectivity or GFP production that was diluted out in the beginning of the dilution series. This phenomenon remains unexplained but was repeatable. The virus lacking endogenous Rev produced with additional 600ng Rev did not show this effect. The viruses were tested in differing GFP reporter cells lines, so it is possible the observation was due to a negative effect on the G11 cells or a difference in the cell lines. The results for both these assays are shown in Figure 37.

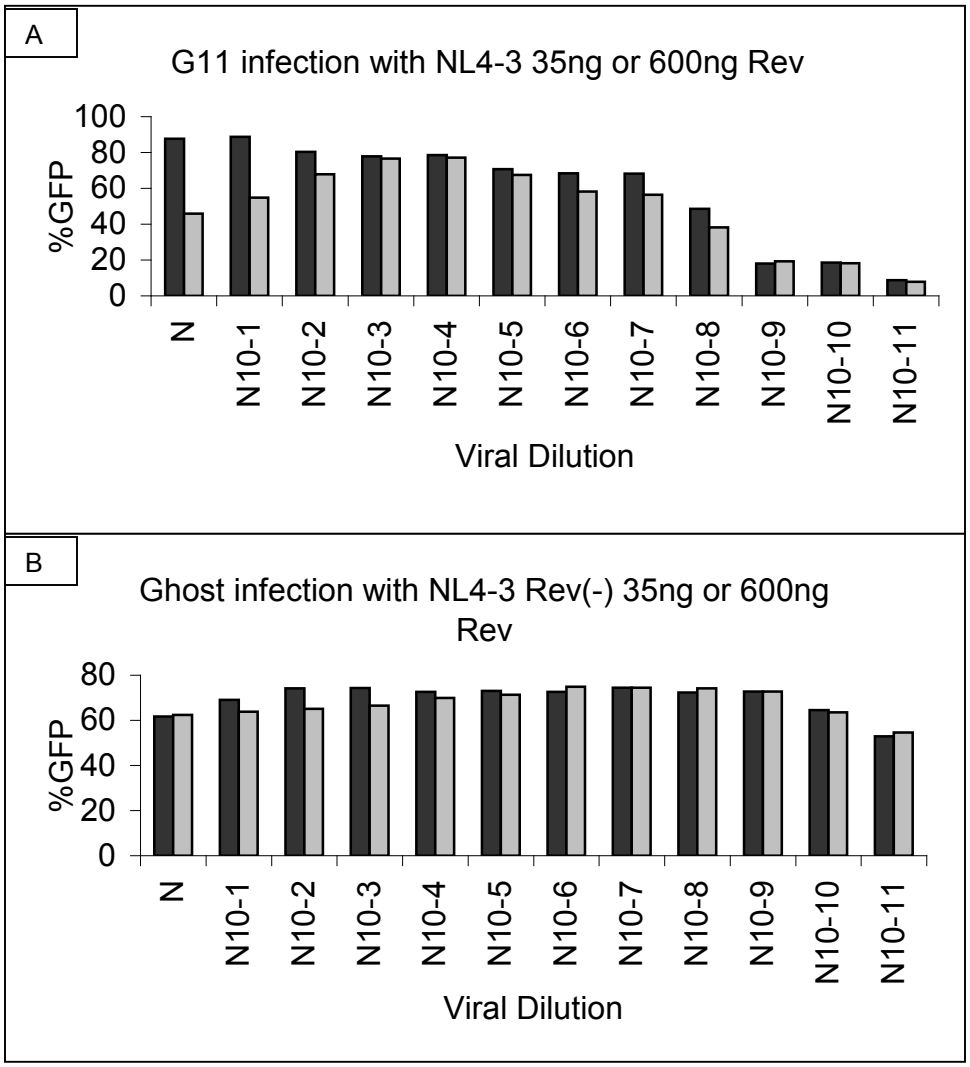


Figure 37. GFP Infectivity Assays

A: G11 cell infection with viral dilutions of NL4-3 virus produced from transfection with additional 35ng or 600ng Rev.

(NL4-3 35ng Rev (black), NL4-3 600ng Rev (gray))

B: Ghost cell infection with viral dilutions of NL4-3 Rev (-) virus produced from transfection with 35ng or 600ng Rev.

(NL4-3 Rev (-) 35 ng Rev (black), NL4-3 Rev (-) 600ng Rev (gray))

Enfuvirtide Resistance (T-20)

The previous work revealed mutations in the RRE that conferred resistance to the Rev-RRE inhibitor compounds. This genetic area of the RRE overlaps known resistance mutations in gp41 for fusion inhibitors such as enfuvirtide (T-20). The majority of T-20 resistance mutations are located in amino acids 36 through 45 of gp41. NL4-3 has a D in gp41 amino acid 36, encoded by an A at nucleotide position 7854, which confers resistance to T-20. Virus with a G at position 7854, conferring a G amino acid in gp41, has been reported to result in sensitivity to T-20 (95). We expected that our Rev-RRE resistant mutant that had the mutation A7854G, conferring D36G in gp41, would be sensitive to T-20. We were uncertain how the other Rev-RRE resistant mutant would respond to T-20, because it had an A at position 7854, conferring a D in gp41, like NL4-3. But it also had an additional change C7836T that conferred A30V in gp41 that had not been previously reported as a resistance mutation.

We wanted to confirm this natural resistance of NL4-3 to T-20, and also determine T-20 resistance patterns of the Rev-RRE inhibitor mutants. To do this we cultured each virus in SupT1 cells in the presence of various concentrations of T-20 (1ng, 0,75ng, 0.5ng, and 0 for control). When we cultured NL4-3 nef (-) in the presence of T-20 we confirmed its resistance. NL4-3 was able to grow virus better that the control in the presence of 0.5ng and 0.75ng T-20, and as well as the control in the highest concentration of 1ng T-20 (Figure 38 Panel A). The Rev-RRE A7854G

resistant variant was sensitive to T-20. Viral growth in the presence of each concentration of T-20 was inhibited, until the end of the growth curve when virus began escape the inhibitory affects in a dose dependent manner (Figure 38 Panel B). The resistant variant selected by 103833 that has a C7836T mutation proved interesting. It demonstrated delayed growth to a similar peak value in the presence of each concentration of T-20, suggesting some sensitivity to T-20, but not to the level as the 104366 selected variant with A7854G (Figure 38 Panel C).

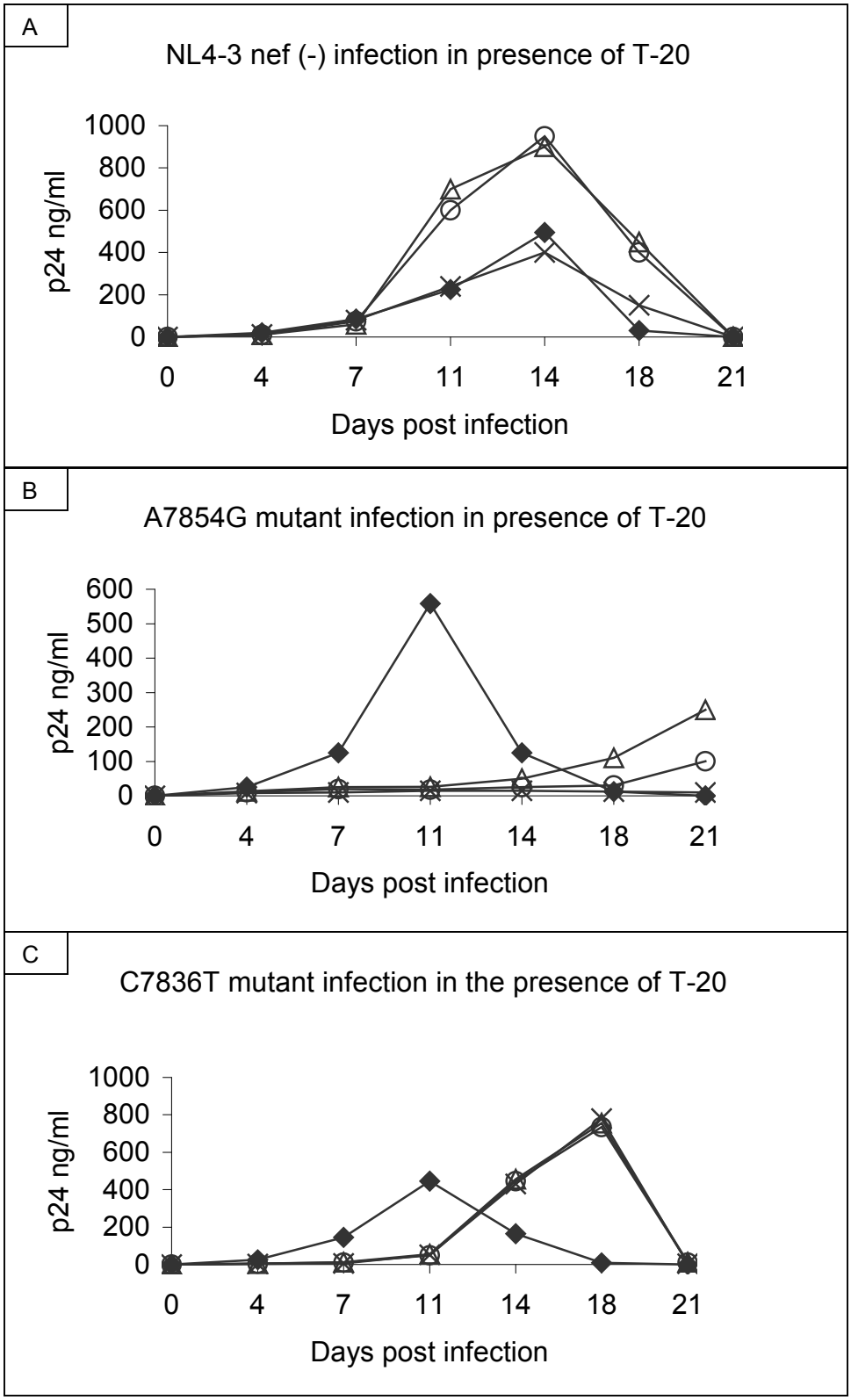

A
NL4-3 nef (-) infection in presence of T-20
p24 ng/ml
1000
800
600
400
200
0
0
4
7
11
14
18
21
Days post infection
B
A7854G mutant infection in presence of T-20
p24 ng/ml
600
500
400
300
200
100
0
0
4
7
11
14
18
21
Days post infection
C
C7836T mutant infection in the presence of T-20
p24 ng/ml
1000
800
600
400
200
0
0
4
7
11
14
18
21
Days post infection

Figure 38. T-20 Resistance Patterns.

NL4-3 and Rev-RRE inhibitor mutant viruses were used to infect SupT1 cells in the presence of enfuvirtide (T-20). ELISA was used to measure p24 at each time point.

A: NL4-3 infection of SupT1 cells in the presence of different concentrations of T-20.

no drug (◆), 0.5ng T-20 (△), 0.75ng T-20 (○),1ng T-20 (×)

B: A7854G mutant infection in the presence of T-20.

no drug (◆), 0.5ng T-20 (△), 0.75ng T-20 (○),1ng T-20 (×)

C: C7836T mutant infection in the presence of T-20.

no drug (◆), 0.5ng T-20 (△), 0.75ng T-20 (○),1ng T-20 (×)

We were interested in how the three viruses would respond to dual pressures of both T-20 and the Rev-RRE inhibitors in order to maintain RRE and Env function. Residue 7854 was of particular interest because of the opposite effect of the selective pressures by the Rev-RRE inhibitors compared to T-20. To study this further we performed long term selection of NL4-3 and the two Rev-RRE inhibitor resistant variants in SupT1 cells, in the presence of both T-20 and a Rev-RRE inhibitor compound (103833 or 104366). Cultures were monitored for viral growth by measuring the p24 by ELISA from the culture supernatants at each splitting. After different time periods, all cultures were able to produce variants that overcame the inhibitory effects of both compounds (Figure 39).

The C7836T (T mutant) virus was able to escape the inhibition in all selection cultures in the shortest time period. Because this virus only had moderate sensitivity to T-20, and resistance to the Rev-RRE inhibitors, it was better able to overcome the pressures of the dual selection. We collected the peak growth of the variant viruses and isolated viral RNA for RTPCR as described. We analyzed the bulk PCR for mutations revealing changes in the RRE, *env*, *tat*, and *vpu* (Table 5).

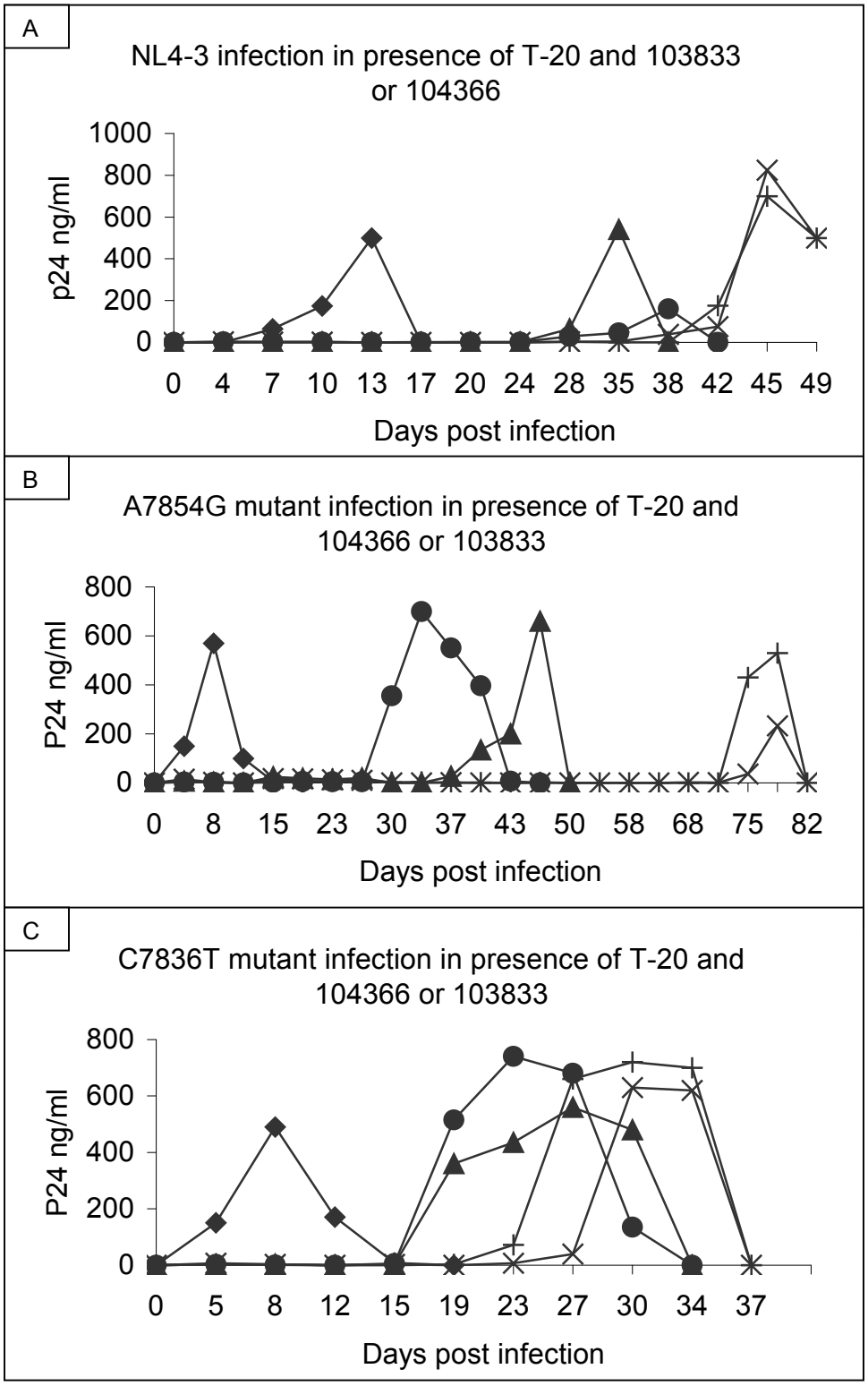

A
NL4-3 infection in presence of T-20 and 103833 or 104366
p24 ng/ml
1000
800
600
400
200
0
0 4 7 10 13 17 20 24 28 35 38 42 45 49
Days post infection
B
A7854G mutant infection in presence of T-20 and 104366 or 103833
P24 ng/ml
800
600
400
200
0
0 8 15 23 30 37 43 50 58 68 75 82
Days post infection
C
C7836T mutant infection in presence of T-20 and 104366 or 103833
P24 ng/ml
800
600
400
200
0
0 5 8 12 15 19 23 27 30 34 37
Days post infection

Figure 39. T-20 and Rev-RRE Inhibitor Dual Selection.

NL4-3 and Rev-RRE Resistant Viruses were used to infect SupT1 cells in the presence of both Rev-RRE inhibitor and Enfuvirtide (T-20) fusion inhibitor. ELISA was used to measure p24 at each time point.

A: NL4-3 dual selection with Rev-RRE inhibitor and T-20.

no drug (◆), 0.75ng T-20 1μM 103833 (×), 0.75ng T-20 0.8μM 104366 (▲), 1ng T-20 1μM 103833 (+), 1ng T-20 0.8μM 104366 (●)

B: A7854G mutant dual selection with Rev-RRE inhibitor and T-20.

no drug (◆), 0.75ng T-20 1μM 103833 (×), 0.75ng T-20 0.8μM 104366 (▲), 1ng T-20 1μM 103833 (+), 1ng T-20 0.8μM 104366 (●)

C: C7836T mutant dual selection with Rev-RRE inhibitor and T-20.

no drug (◆), 0.75ng T-20 1μM 103833 (×), 0.75ng T-20 0.8μM 104366 (▲), 1ng T-20 1μM 103833 (+), 1ng T-20 0.8μM 104366 (●)

Table 5. Mutations identified in Sequenced RTPCR product of dual selected variants (Rev-RRE inhibitor and Enfuvirtide (T-20)).

Culture Fig. 39	VIRUS	SELECTION	ORIGINAL RRE	RRE CHANGES	OTHER CHANGES
Panel C ▲	T mutant	0.75ng/ml T-20 0.8uM 104366	C→T 7836, stem loop IIC	Back to C @ 7836, stem loop IIC A→G 7854, stem loop IIC A→G 7936, stem loop V	5842 tat 1 G→C (GAT→CAT) Asp to His 6773 gp120 V2 G→A (GAT→AAT) Asp to Asn 7741 gp 120 C term A→G (GAA→GAG) E 8345 and 8347 gp41 G→A and A→C (GGA to AGC) Gly to Ser 8358 gp41 T→A (TTA→TAA) L to premature stop in env before rev 2nd exon
Panel C ●	T mutant	1ng/ml T-20 0.8uM 104366	C→T 7836, stem loop IIC	Back to C @ 7836, stem loop IIC A→G 7854, stem loop IIC AG Mix 7936	5842 tat 1 G→C (GAT→CAT) Asp to His 7741 gp 120 C term A→G (GAA→GAG) E no change
Panel C ×	T mutant	0.75ng/ml T-20 1uM 103833	C→T 7836, stem loop IIC	Back to C @ 7836, stem loop IIC A→G 7854, stem loop IIC A→G 7936, stem loop V	
Panel C ＋	T mutant	1ng/ml T-20 1uM 103833	C→T 7836, stem loop IIC	Back to C @ 7836, stem loop IIC A→G 7854, stem loop IIC A→G 7936, stem loop V	5842 tat 1 G→C (GAT→CAT) Asp to His 7741 gp 120 C term A→G (GAA→GAG) E no change

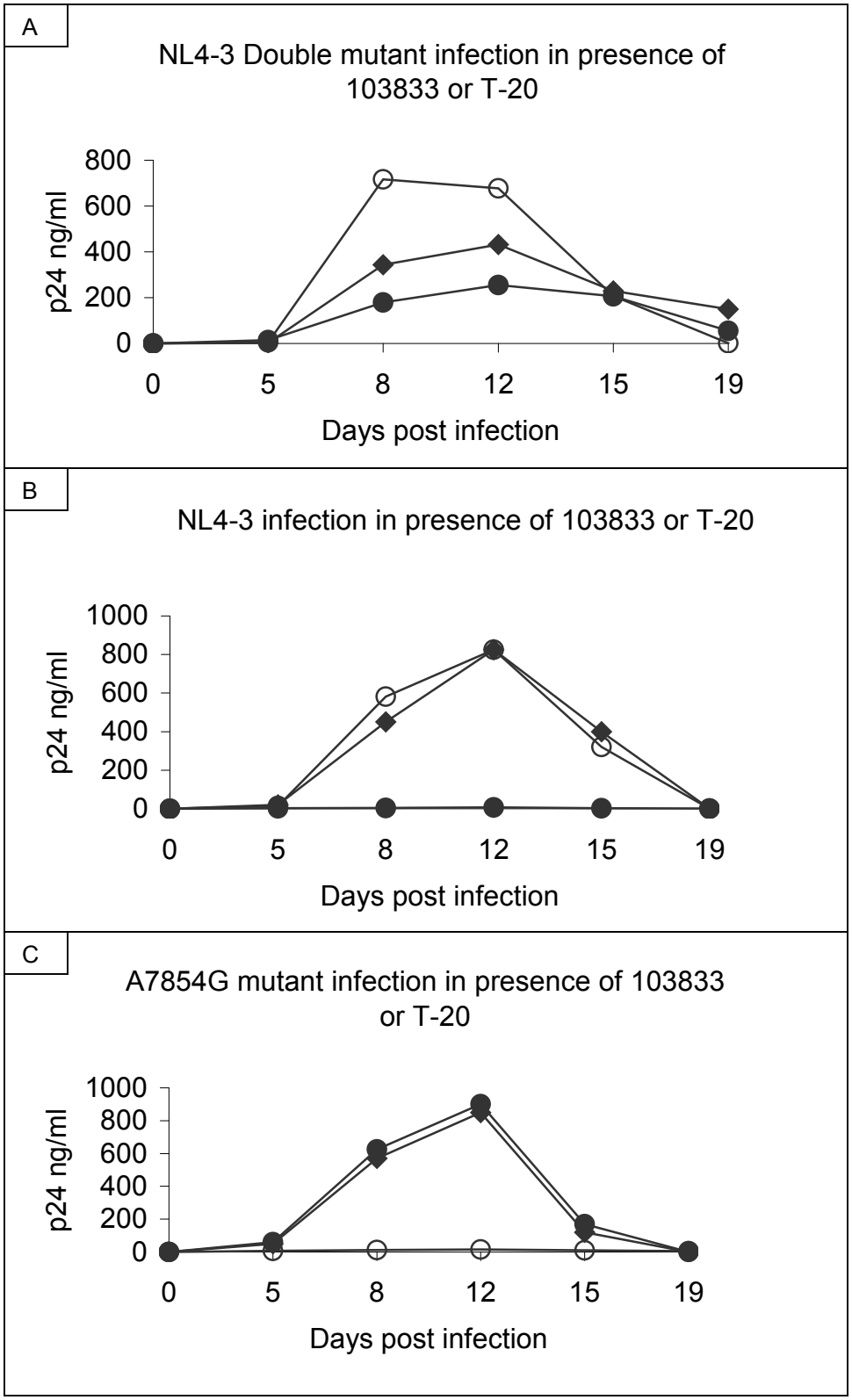
Culture Fig. 39	VIRUS	SELECTION	ORIGINAL RRE	RRE CHANGES	OTHER CHANGES
Panel B ▲	G mutant	0.75ng/ml T-20 0.8uM 104366	A→G 7854, stem loop IIC	A→G 7936, stem loop V	
Panel B ●	G mutant	1ng/ml T-20 0.8uM 104366	A→G 7854, stem loop IIC	A→G 7936, stem loop V	
Panel B ＋	G mutant	1ng/ml T-20 1uM 103833	A→G 7854, stem loop IIC	Back to A @ 7854, stem loop IIC TC mix C→T 7836, stem loop IIC	5916 tat1 G→A (AAG→AAA) K 6063 start vpu AG mix G→A (ATG→ATA) start to Ile 6773 gp120 V2 G→A (GAT→AAT) Asp to Asn

Culture Fig. 39	VIRUS	SELECTION	ORIGINAL RRE	RRE CHANGES	OTHER CHANGES
Panel A ▲	NL4-3	0.75ng/ml T-20 0.8uM 104366	NL4-3 RRE	A→G 7854, stem loop IIC A→G 7936, stem loop V	
Panel A ×	NL4-3	0.75ng/ml T-20 1uM 103833	NL4-3 RRE	TC mix C→T 7836, stem loop IIC	5916 tat1 G→A (AAG→AAA) K 6063 start vpu AG mix G→A (ATG→ATA) start to Ile 6773 gp120 V2 G→A (GAT→AAT) Asp to Asn 7741 gp 120 C term A→G (GAA→GAG) E
Panel A ＋	NL4-3	1ng/ml T-20 1uM 103833	NL4-3 RRE	A→G 7854, stem loop IIC A→G 7936, stem loop V	

We used the bulk PCR to generate TOPO Clones as described in the methods. Clones were sequenced and analyzed for the mutations identified in the bulk PCR products. Only clones with the mutations A7854G in stem loop IIC and A7936G in stem loop V of the RRE were isolated. The other mutations in *tat, env,* and *vpu* were not identified by this method, and have not yet been pursued. Because all the clones analyzed contained the two A to G changes, we reasoned these mutations were present at a high frequency and could be important toward dual resistance. We built the mutations back into the NL4-3 wild type backbone as described in the methods. The resultant virus was used to infect SupT1 cells in the presence of both compounds (Figure 40 Panel D). The virus was able to grow to a similar peak level even in the presence of both T-20 and 103833 or 104366 compounds.

The double mutant virus was also cultured in the presence of T-20 or 10μM 103833 to determine the influences of each separate compound (Figure 40 Panel A). For comparison the NL4-3 and A7854G mutant viruses were grown in tandem under the same conditions (Figure 40 Panel B and C). The double mutant virus grew better in the presence of T-20 than it did in the absence of compound. The virus showed growth above that demonstrated by the wild type NL4-3 in the presence of 103833, but not to the level previously demonstrated by the 103833 or 104366 selected variants. This virus was interesting in that it contained the A7854G mutation that was previously shown to confer resistance to the

Rev-RRE inhibitor compounds but sensitivity to T-20. But it also contained a silent mutation A7936G. This nucleotide change does not alter the amino acid sequence of gp41, but in some way allows for T-20 resistance. This change also appeared to decrease the resistance to the Rev-RRE inhibitor 103833. More research is needed into the importance of stem loop V and its interaction with cellular proteins that affect Rev-RRE and Env function.


A
NL4-3 Double mutant infection in presence of 103833 or T-20
p24 ng/ml
800
600
400
200
0
0
5
8
12
15
19
Days post infection
B
NL4-3 infection in presence of 103833 or T-20
p24 ng/ml
1000
800
600
400
200
0
0
5
8
12
15
19
Days post infection
C
A7854G mutant infection in presence of 103833 or T-20
p24 ng/ml
1000
800
600
400
200
0
0
5
8
12
15
19
Days post infection

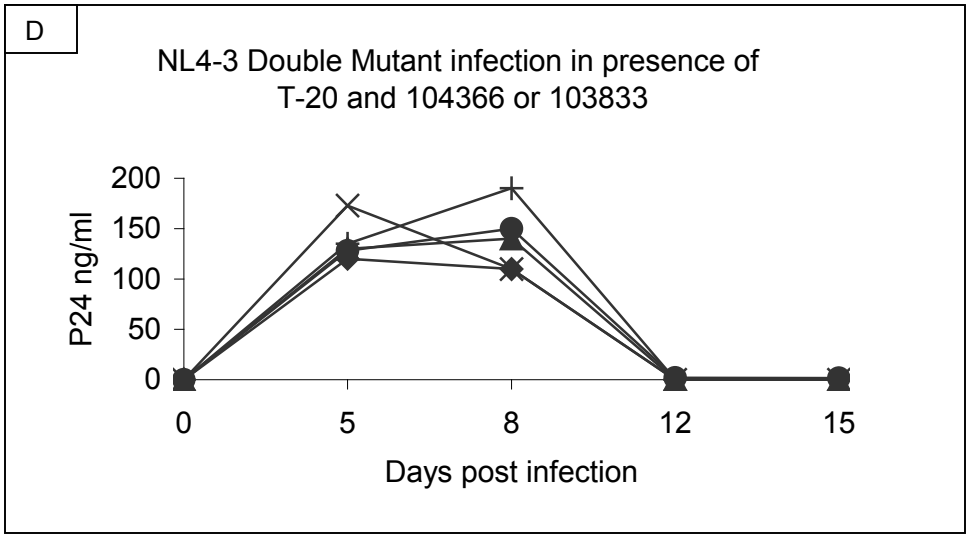


Figure 40. Dual Selected Variant Compared to NL4-3.

ELISA used to measure p24 at each time point.

A: Double selected (T-20 and Rev-RRE inhibitor) mutant growth in SupT1 cells in the presence of 103833 or T-20.

no drug (◆), 10μM 103833 (●), 1ng T-20 (○)

B: NL4-3 infection of SupT1 cells in the presence of 103833 or T-20.

no drug (◆), 10μM 103833 (●), 1ng T-20 (○)

C: A7854G mutant (selected by 104366) growth in SupT1 cells in the presence of 103833 or T-20.

no drug (◆), 10μM 103833 (●), 1ng T-20 (○)

D: Double selected (T-20 and Rev-RRE inhibitor) mutant growth in SupT1 cells with no drug (◆), 0.75ng T-20 1μM 103833 (×), 0.75ng T-20 0.8μM 104366 (▲), 1ng T-20 1μM 103833 (+), 1ng T-20 0.8μM 104366 (●).

Enfuvirtide and R73

Interestingly R73 contains the same RRE sequence (A7854G, A7936G) as the NL4-3 variant resistant to both T-20 and the Rev-RRE inhibitor compounds. We were interested how this virus, with a different genetic background from NL4-3, would respond to T-20. We cultured R73 in SupT1 cells in the presence of varying T-20 concentrations (Figure 41). R73 was naturally resistant, and grew better at the lower concentration than it did without compound. The lower concentration tested for R73 was actually 10x higher that the concentration used to inhibit NL4-3. The culture exposed to the higher concentration of T-20 (100ng) was inhibited until Day 31. At this concentration we selected for a R73 resistant variant to T-20 (100x higher that the concentration used to inhibit NL4-3) (Figure 41). We collected virus from the peak of this culture and isolated viral RNA. We used RTPCR to amplify fragments of interest as previously shown in Figure 34. Using DNA sequencing of the bulk PCR as described in the methods, mutations were identified in the RRE of the T-20 resistant variant. The mutations were previously identified enfuvirtide resistance mutations in the HR1 of gp41 (Table 6).

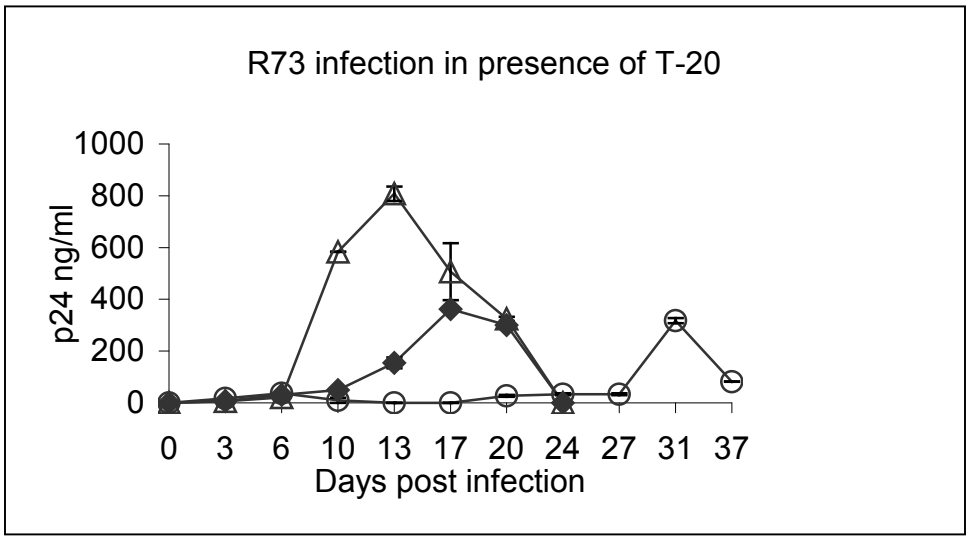


Figure 41. R73 Resistance Pattern and T-20 Selection

R73 infection of SupT1 cells in the presence of T-20.

Viral input of 10ng used for infection of 5 million cells.

ELISA used to measure p24 at each time point.

Curves represent duplicate cultures with error bars shown: no drug (◆), 10ng T-20 (△), 100ng T-20 (○)

Culture Fig. 41	VIRUS	SELECTION	ORIGINAL RRE	RRE CHANGES	OTHER CHANGES
O culture	R73 Day 31	100ng T-20	A→G 7854, stem loop IIC A→G 7936, stem loop V	7858 A→G ATA to ATG Ile to Met aa 37 gp41, 7875 A→G AAT to AGT Asn to Ser aa 43 gp 41	NONE

Table 6. Mutations identified by Selection of R73 to T-20

Envelope expression in various viruses

The Rev-RRE pathway controls the level of envelope message that is expressed, and changes in the sequence of gp41 (overlapped by the RRE) also can affect the function of the envelope trimer. The variants identified in the selection studies had changes in both the RRE and envelope region, so we were interested in determining the Env expression levels in each of these viruses. We performed infections of SupT1 cells with the various viruses. As described in the methods we prevented further rounds of infection by the use of the Protease inhibitor Saquinavir, preventing viral maturation. We collected the cells and used them for western blots for Env. The Env level was normalized to both cellular Beta Tubulin and the p55 in the cell. We quantitated the blots using an Odyssey infrared scanner (LiCor, Inc.). The analysis showed only slight differences in steady state levels of Env between viruses in first round SupT1 infections. The Rev-RRE resistant variants produced the most Env, R73 and the double selected variant produced a middle value of Env, and the NL4-3 RRE containing viruses produced the least steady state Env. The R73 mutants produced similar Env steady state levels as R73. But the error associated with the combined experiments was large making it difficult to determine if the observed trends in steady state Env levels were significant (data not shown). Other experiments that address not only steady state Env, but also the function of Env in the various viruses need to be performed to address this issue.

Chapter 4

Discussion

Antiviral Compounds

Efforts to identify compounds that can effectively inhibit HIV-1, continues to be a major focus of AIDS research. Therapeutic agents have historically targeted viral enzymatic proteins, but efforts have expanded therapies to target other essential viral processes like fusion and viral entry. Based on previous work, we were interested in exploring compounds that could interfere with the Rev-RRE pathway.

At this point no therapies are clinically available that target the Rev-RRE pathway. Some of the current inhibitors being studied include binding mimetics. One example uses RNA aptamers that decoy the Rev protein from the RRE target, thereby preventing binding and function (79, 138). Other researchers are studying α-helical peptidomimetic inhibitors that bind the RRE, and prevent Rev binding and function (116). One of the original examples of a peptide Rev inhibitor is RevM10. This peptide is a transdominant inhibitor that is able to bind the RRE, but cannot export due to a defective NES (60). RevM10 is a competitive inhibitor of Rev. Similar peptides are currently being studied in clinical trials. More novel approaches use either anti-sense RNA or miRNA to target the mRNA cargo of the Rev-RRE pathway (149). The anti-sense approach, although not fully understood, is exciting and involves the intracelluar

“immunization” of HIV target cells with packagable inhibitor RNAs that can be transported to other host cells. The effectiveness and safety of this novel approach is currently being evaluated.

Chemicals that interfere with the high affinity binding of Rev to the RRE (Neomycin, diphenylfurans) have been identified (134, 184). However, due to their toxicity, these compounds cannot be used clinically in humans to treat HIV infection. Leptomycin B also effectively inhibits Rev-RRE function by inhibiting Crm1, a Rev cofactor (9). But, again, this compound is too toxic for systemic use in humans. Aromatic heterocyclic compounds show promise for potential Rev-RRE inhibitor compounds (180). These compounds have been previously shown to interfere with Rev-RRE complex formation and function (108). Large libraries of heterocyclic compounds exist from the development of other inhibitor molecules. Our laboratory has pursued the viability of small molecules as inhibitors of Rev-RRE function.

Our laboratory had identified heterocyclic compounds shown to inhibit HIV-1 particle production. Approximately 40,000 compounds from a commercially available library were screened at Message Pharmaceuticals in 5BD.1 cells developed at our laboratory, resulting in 192 compounds with greater than 50% inhibition. These hits were further tested with 3 and 6 step dose response assays, and MTS-based toxicity assays to generate therapeutic index (TI) values for the compounds (TC50/IC50). Only 12 compounds were selected as possible candidate

molecules. The 5BD.1 cells produce viral particles in a Rev dependent manner. The 12 compounds were further explored for anti-HIV and anti-Rev activity.

With our collaborators at Southern Research Institute, we tested the compounds in Primary Blood Mononuclear Cells (PBMCs) infected with NL4-3 (X4 tropic Subtype B Laboratory HIV-1) and BR/93/021 (R5 tropic Subtype B HIV-1 patient isolate). Supernatant from the cultures was analyzed for reverse transcriptase (RT) activity. Cytotoxicity was measured by addition of MTS to the plates for determination of cell viability (MTS dye reduction). The compounds used in this research inhibited the growth of both NL4-3 and BR/93/021 with minimal toxicity.

Rev Specificity of the Compounds

Our laboratory through collaboration with Southern Research Institute tested the specificity of the compounds for the Rev-RRE pathway. Cells containing a Rev-dependent Renilla luciferase reporter and a Rev-independent Firefly luciferase reporter were exposed to decreasing ½ log increments of compound in 96 well plates. The therapeutic index of this assay was measured by comparing the inhibition of the Rev-dependent Renilla versus the Firefly luciferase that served as a control. This assay demonstrated the specificity of the compounds for the Rev-RRE pathway.

In comparison cells containing a dual reporter system, Renilla luciferase, produced with HIV-1 Tat, or Firefly luciferase, produced from normal mRNA that does not require Tat, were exposed to decreasing ½

log increments of compound in 96 well plates. The therapeutic index of this assay was measured by comparing the inhibition of the Rev-dependent Renilla versus Firefly luciferase that served as a control. The compounds did not show specificity for Tat.

The compounds were also tested in a U1 latency assay. U1 cells were induced with 5ng/ml TNF-alpha in the presence of concentrations of the compounds. Cultures were incubated for 3 days and the supernatants harvested for reverse transcriptase analysis. Toxicity was determined by MTS dye reduction. The compounds were tested in this assay at a time point after integration during viral replication to viral release. This is the point in the viral life cycle where the Rev-RRE pathway is known to function. The compounds were shown to produce favorable therapeutic index values in this assay.

To further use the U1 assay to test for specificity of the compounds for the Rev-RRE pathway, we tested the U1 cells in western blots for p24 and Nef proteins. Cells were either uninduced or had either been induced in media without compound, or in the presence of 103833 or 104366. The compounds did not significantly affect Nef levels, whereas they greatly reduced Rev-dependent p24 and p55. Again these results showed the specificity of the compounds for the Rev-RRE pathway. This result for the compounds was mirrored in western blots of 293T cells transfected with NL4-3 provirus. The transfection assay measures the time frame from proviral replication to viral release, similar to the time point of induction of

the latent provirus to viral release in the U1 cells. Again p24 and p55 proteins were selectively reduced compared to Nef.

Our laboratory tested pCMVGagPol constructs containing either the RRE or MPMV CTE in transfection assays in the presence of increasing concentrations of the compounds. An in house ELISA was used to measure the level of p24 produced by the constructs after 72 hours. The CTE constructs were Rev-independent, compared to the RRE constructs that required Rev supplied in trans for expression. The assay showed selective reduction of the expression of the Rev-RRE constructs by the compounds in a dose dependent manner.

Our laboratory also tested the compounds using in vitro gel shift experiments. Using a 32P-UTP radioactive RRE RNA probe, we visualized and quantified Rev-RRE multimeric complexes using a phosphoimager. Although the compounds previously showed specificity for the Rev-RRE pathway, no difference in gel shift was evident due to the compounds in this assay. In comparison, neomycin, a known inhibitor of Rev-RRE binding, completely inhibited a shift from forming. This result suggests that the compounds do not inhibit high affinity binding in this assay.

Previous research using aromatic compounds showed inhibition of Rev-RRE complex formation (185). Our collaborators at the National Cancer Institute have been studying the compounds, using Mass Spectrometry, to determine interactions with the Rev protein or RRE RNA.

At this point they have determined that the compounds do not interact with Rev. Further research in vitro is being performed to determine the interactions of the compounds with the RRE RNA.

Selection and Characterization of Resistance Mutations

The idea of reverse genetics has long been demonstrated as an effective tool to study genetic function. A similar concept can also be used to study the mechanism of action of anti-retrovirals. If the virus is able to become resistant to the effects of a compound, then this molecule must be effectively inhibiting the virus prior to the development of resistance. Understanding how the virus is able to change, in order to overcome the inhibition, allows for greater understanding of the viral molecular physiology.

We used selection experiments to better understand the Rev-RRE pathway. If the compounds were inhibiting Rev-RRE function, the virus would have to overcome the causes of the inhibition. Studying resistant variants would provide further understanding of the Rev-RRE pathway that is essential for viral replication. We hypothesized that the virus could alter several areas to cause resistance: The virus could alter *rev,* allowing for increased or altered protein expression that would improve Rev-RRE function; The RRE structure could be altered to improve Rev-RRE association either in the primary binding site or through improved multimerization; RRE structural changes could positively affect cellular protein associations necessary for Rev-RRE function; Env function could

be improved to increase viral infectivity in the culture; or Vpu expression could be decreased or eliminated thereby increasing Env levels. We decided to concentrate on identifying mutations in those genetic areas (*rev, vpu, env,* RRE) covering approximately 1/3 of the viral genome. Because changes in other viral genetic areas that were not studied could also cause resistance, we used molecular techniques to build the mutations identified in our studied fragments back into the wild type virus backbone. Therefore, only the mutations of interest were used to test for the resistant phenotype in the virus.

As expected, we did recover resistant variants to both 104366 and 103833. Interestingly, the variants were cross resistant to the other compound. This suggested a similar mechanism of action of the compounds and possibly a similar mechanism of resistance. The mutations identified to confer the resistant phenotype were in the RRE. To our excitement the variants selected by two separate compounds caused the same structural change in the base of stem loop IIC of the RRE. We hypothesized elongation of the stem stabilized the RRE or protein association with the RRE. The mutations also changed amino acids in gp41 of Env.

Because the mutations caused both structural changes in the RRE and amino acid changes in gp41, we wanted to separate the two possible contributors to resistance. We tested the compounds in transient transfections of 293T cells using proviral constructs containing either the

NL4-3 or resistance mutations RREs. This assay measures a time frame from proviral replication to viral release. Because the 293T cells are not infected, the virus produced does not depend on gp41. By measuring p24 for the level of viral particles produced after 72 hours, we were able to determine if the compounds produced the same pattern shown in viral culture. We concluded the mutant RREs were able to produce virus even in the presence of the compounds, but the wild type RRE was inhibited. This suggested the structural changes in the mutated RREs increased Rev-RRE pathway function to overcome the effects of the compounds regardless of gp41 amino acid sequence.

This one experiment reinforced how important the structure of the RRE could be toward Rev-RRE pathway function. Most studies, in general, have neglected the importance of contributions by the RRE. The most emphasis is placed on the viral proteins, such as Env, and the changes in amino acid sequences that could influence the function of the proteins (119). Because the Rev-RRE pathway involves the association of viral and cellular proteins with a RNA structure, the RNA must be considered with just as much regard. Unfortunately, HIV researchers have often neglected to take into account the genetic background of viruses or viral components studied. If such a minimal change in the RRE can have such a large effect on the virus, we need to be more discriminating in our efforts.

We also performed further experiments to separate the possible contributors to the resistant phenotype in the context of a viral culture. Using site directed mutagenesis, we disrupted the RRE structure but left the amino acid changes intact conferred by the resistance mutations. The result was virus that was no longer resistant to the compounds. The disruption in the RRE structure caused the virus to behave like the sensitive wild type parent. Therefore, we could conclude that the structural changes in the RRE were the important component for resistance. The amino acid sequence of gp41 may have improved Env function in viral culture, but the changes were not sufficient enough to cause resistance.

This is an important concept for HIV biology. The RRE structure has been studied primarily as a high affinity binding site for Rev (85), but our mutations were outside this area. We hypothesized that the changes stabilized the base of the stem IIC. In fact, our collaborators at the National Cancer Institute confirmed the formation of the additional nucleotide binding pair at the base of stem loop IIC of the RRE in the mutant RREs. The structural changes could have important consequences for Rev-RRE multimerization or interaction with host cell proteins necessary for pathway function.

We were interested if the structural changes improved Rev-RRE function, so we then tested the RREs with Rev titrations and were excited to discover that the mutant RREs were able to function at lower Rev levels

than the wild type RRE. This was especially apparent in the GagPol construct transfections, where the mutant RREs produced viral particles at Rev levels ~20x lower than the wild type. So a single nucleotide change in the RRE was able to dramatically affect Rev-RRE function.

Unexpectedly, we were able to show a negative effect on viral production for the NL4-3 proviral transfections at certain Rev levels. This biphasic effect was not observed for the GagPol constructs or the R73 provirus. In addition to an altered genetic background, R73 lacks Vpu, Vpr, and Nef. The GagPol constructs also lack these proteins in addition to other genetic areas of the virus. We hypothesized that at certain trans Rev levels, the NL4-3 proviruses were induced to express accessory proteins in a way that was detrimental to the transfection system. Vpr is known to cause cellular apoptosis, affect the cell cycle, and affect cellular transcription (104). This viral protein could be the cause of the negative effect observed. Why this effect was seen at certain trans Rev levels and not others remains a question for future research. Rev titration experiments comparing NL4-3 proviruses with deletions separately in each accessory protein could show the contributor to this biphasic response. The expression of the accessory proteins could also be measured. These experiments could determine the importance of not just expression of each accessory protein, but of the amounts and timing of the expression of these proteins to the virus.

Except for NL4-3, most of the HIV-1 Clade B viruses sequenced to date contain a G at RRE nucleotide position 7854. NL4-3 is, therefore, an outlier. We hypothesize that naturally occurring HIV-1 Clade B viruses could be able to function at lower Rev levels than NL4-3. This change, causing a G at amino acid 36 of gp41, has been previously documented to increase the fitness of HIV-1 in the absence of any selection (95). This was also documented subjectively during my viral growth experiments. The virus was able to grow to a peak level of viral production faster than the NL4-3 virus. Also, reversion experiments failed to cause the resistant variant to revert to the NL4-3 RRE sequence. After ten passages of this virus in the absence of selective pressure, the resultant variant still maintained resistant to the Rev-RRE inhibitor compounds. We hypothesize that this change is beneficial for viral fitness, and that this correlates to improved Rev-RRE function.

It has been previously reported that this change can also improve viral binding and fusion (95), but we demonstrated, by our site mutagenesis experiments, that the amino acid change in gp41 alone could not overcome the inhibition of the Rev-RRE compounds. Improved fusion did not in this case provide the edge the virus needed to escape the effects of the compounds. Increasing the number of viral particles entering the cell did not overwhelm the compounds. This suggests the compounds are not competitive inhibitors.

RRE and gp41

Studies of fusion inhibitor resistance have identified amino acids in gp41 that most frequently change to overcome the inhibitors. Over 90% of mutations from studied clinical cohorts were in amino acids 36-45 (150). These amino acids are coded from RNA that overlaps the location of the RRE stem loops IIC and III. In fact, as previously discussed, the entire RRE is contained within the gp41 coding sequence (119). So changes that occur in this genetic area would affect both Env and RRE function. We were interested in the possibilities of using Rev-RRE inhibitors with fusion inhibitors as combined therapies for HIV.

Because the RRE mutations we identified overlap the coding sequence for gp41, we were interested in double selection experiments with the Rev-RRE compounds and the fusion inhibitor enfuvirtide (T-20) (119). Amino acid 36 has been shown to be important for T-20 resistance in previous research. When nucleotide 7854 is an A, like NL4-3, the virus is resistant to T-20. But when it is a G, like the Rev-RRE resistant variant, the virus is sensitive to T-20 (117). There are opposite selection pressure by the two compounds on this particular residue. This certainly raises the possibility of using the Rev-RRE inhibitors and fusion inhibitors as "convergent" therapies. The virus may not be able to escape as easily compounds targeting the same area but in different ways.

These resistance patterns to T-20 of NL4-3 and the A7854G mutant were confirmed in my research by viral growth curves. The other resistant

variant with a T at RRE nucleotide 7836 showed a delayed growth pattern in the presence of the T-20. This virus contains an A at position 7854 like NL4-3, so we expected the virus could be resistant, but instead it was somewhat affected by the compound. The change to amino acid 30 from A to V may have some detriment to T-20 resistance.

Interestingly, the double selection of NL4-3 and both Rev-RRE resistant variants produced double resistant variants with the same RRE sequence. This virus contained the G at position 7854 of stem loop IIC conferring Rev-RRE resistance (but T-20 sensitivity), and an additional G at position 7936 of stem loop V. That mutation did not change the gp41 amino acid sequence (silent mutation). But when these mutations were tested in the wild type backbone, they were shown to confer both T-20 and Rev-RRE resistance. The level of viral of growth of this virus, in the presence of only the Rev-RRE compounds, was less or delayed compared to that that of virus with only a change in stem loop IIC (either position 7836 or 7854). The stem loop V change appeared to have a diminishing effect on Rev-RRE resistance. This finding also shed light on how other viruses containing both these RRE changes tested sensitive to the compounds in the PBMC assays performed at Southern Research Institute. If the compounds only marginally inhibited these viruses, a delayed growth pattern would not have been seen in the time frame of the dose response assays.

For example, the Brazilian isolate BR/93/021 has the A7854G change in RRE stem loop IIC as compared to NL4-3. This change was identified in my research as a Rev-RRE compound resistance mutation, but in this assay the isolate was shown sensitive to the compounds. BR/93/021 also has an additional A7936G change in RRE stem loop V that was identified in my research during dual selection with T-20 and 103833. This change was shown to allow for T-20 resistance, but diminish resistance to 103833 or 104366. The PBMC infections were not performed over a long enough time, only 7 days, to determine if BR/93/021 could break through the effects of the compounds. The dual resistant mutant and R73 viruses in my research that contain both these changes in their RREs, often showed delayed growth in the presence of the compounds, a week and a half to two weeks after infection. If the PBMC assay were taken out to this time point, the Brazilian isolate may have also broken through the effects of the compounds. It is important to note, however, the PBMC assay and T cell line cultures are performed at different conditions that also affect the response of viruses to the compounds.

Why this change in stem loop V could confer T-20 resistance without altering the amino acid sequence of gp41 is still not clear. As stated before the majority of known T-20 resistance mutations are due to changes in amino acids 36-45 of gp41. We hypothesize this silent change A7936G affects the interaction of stem loop V with cellular proteins that

are involved in Rev-RRE function. This change could affect how *env* is expressed and Env function. T-20 is a competitive inhibitor that can be overwhelmed at a set concentration by more infectious virus entering the cell. Improving Env function is a common explained mechanism for T-20 resistance.

Stem loop V has been reported to have minimal effects on RRE function (88). But other studies conflict showing that disruption of the stem results in a nonfunctional RRE. Oligonucleotides directed against stem loop V have been shown more active at inhibiting Rev-RRE function than those directed at stem loop II (178). In HTLV-1, stem loop V serves as the primary binding site of Rex, the homolog of HIV-1 Rev (22). Although not a primary binding site for Rev, stem loop V may have important protein interactions that are necessary for the Rev-RRE pathway. Finally the tertiary structure of the RRE may associate stem loop V with the Rev-RRE complex in a yet undetermined way that is important for pathway function.

R73

Our work up until this point focused on the NL4-3 laboratory strain of HIV. We also tested a separate virus containing a different genetic background, R73 an HXB-2 like virus, in the presence of the Rev-RRE compounds or T-20. R73 interestingly has the same RRE sequence as the NL4-3 double selected variant. R73 also lacks Vpu, Vpr, and Nef. In my research R73 tested naturally resistant to T-20. R73 also

demonstrated less or delayed growth in the presence of the Rev-RRE inhibitors. It was interesting to us that the R73 virus with A7854G and A7936G in the RRE showed similar patterns in culture to the NL4-3 virus with these RRE changes. These results suggested to us that these changes had a relevant effect that may be seen in other HIV viruses. R73 also has other changes that would affect the viral response in culture. Because R73 lacks Vpu, we inferred it could produce more Env than NL4-3. But R73 also has some changes in the amino acid sequence of Env, which could also affect the fitness of the virus in a yet undetermined way. More work is necessary to determine the Env function in these respective viruses. R73 also lacks Vpr. A reduction in T cell cytopathic effects and death was apparent during light microscopy study of the cultures. The dynamics between the T cells and R73 virus were different compared to NL4-3 cultures. This variation may of affected the response of the virus to the compounds.

To study the virus further, we selected for resistant R73 variants to T-20 and 103833. The T-20 selected R73 acquired additional mutations in the RRE that corresponded to gp41 amino acids 37 and 43. Both were previously identified T-20 resistance mutations (136). The 103833 selected R73 did not change the RRE, but acquired additional changes in gp120 of Env. Two changes at position 6402 and 7295 conferred resistance to 103833 when built back into the wild type R73 background. The mutation A6402C, (GCA to GCC), was a silent mutation leaving an A

in the amino acid sequence of gp120 in the 1st conserved region (C1) amino acid position 48. The mutation A7295C, (GAT to GCT), caused a D346A change in the amino acid sequence of gp120 in the 3rd conserved region (C3) interestingly in a HLA epitope.

It is undetermined why both these mutations together were able to confer resistance to 103833. The R73 wild type parent had only marginal sensitivity to 103833. It was able to grow only after 2 weeks of exposure with no identified mutations. NL4-3 never broke through inhibition by 103833, and required mutation of the RRE to resume viral replication. So we proposed that improved Env function in this case could have given R73 the fitness necessary to overcome 103833.

The changes noted in the R73 mutant in gp120 may also affect more than Env. Splicing could be affected allowing for improved *rev* expression and Rev-RRE function. The mutations are not in areas of known splicing enhancers or silencers, but more research is necessary to determine if either of these mutations positively affect splicing of *rev* (35).

We performed initial experiments to study Env in the NL4-3 and R73 viruses. We tested the steady state Env levels in the infected T cells of first round infections of the different viruses by western blot. Env was normalized to cellular beta tubulin and viral p55. Because of the error of the combined blots performed, significant differences between the viruses could not be determined. The experiments would need to be repeated to determine if the observed trend was relevant (Rev-RRE resistant

variants>R73, R73 Mutants, and NL4-3 Double resistant variant>NL4-3). Other experiments that address not only Env levels, but also Env function should also be performed. The trend however is interesting, because it directly correlates to Rev-RRE function, and inversely correlates to inhibition by the Rev-RRE inhibitor compounds. No significant differences were discernable between the R73 mutants and R73 steady state Env levels in first round infections as measured by western blot. More research would be necessary to determine the effects of these mutations on the function of the Env trimer. We really need to determine what amounts of Env are necessary for function, and how large of a change makes a difference towards function. Then we need to be able to associate changes in Rev-RRE function with the expression of Env, and what that actually means for the virus. Research of the Rev-RRE pathway and Env should be coupled to provide further insight about HIV.

Overall, this research has emphasized that Rev-RRE function is an important targetable component of the life of HIV-1. The Rev-RRE compounds described are potent inhibitors of the Rev-RRE pathway that are only overcome when Rev-RRE function is optimized. Potentially these compounds could be used in combination with inhibitors of gp41 or for salvage of fusion inhibitor resistance. More importantly the compounds have given us key insight into natural HIV Rev levels of HIV-1 Clade B and the Rev-RRE pathway. Research should also be expanded to other HIV-1 Clades and HIV-2.

More research is needed to understand the importance and interactions of the RRE stem loop V. Also Rev-RRE structural studies should be expanded to include the entire RRE structure. In order to target the Rev-RRE pathway, we need to increase our knowledge of how Rev interacts with the RRE outside the high affinity binding site, and how the Rev-RRE complex interacts with cellular proteins. With this increased knowledge we could better target this essential pathway for the virus in potential anti-HIV therapies.

In summary, this research demonstrates how generation of viral variants can be a powerful tool to study viral functions and interactions with inhibitory compounds. The virus selects for variants that can replicate and survive at the fittest level possible. How and where these mutations occur can point research towards mechanisms. These resistance findings can also be applied in future research concerning natural resistance.

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