MULTI-STEP INHIBITION EXPLAINS HIV-1 PROTEASE INHIBITOR PHARMACODYNAMICS AND RESISTANCE

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ABSTRACT

HIV-1 protease inhibitors (PIs) are among the most effective antiretroviral drugs due to highly cooperative dose-response curves that are not explained by current pharmacodynamic theory. Another unresolved problem affecting the clinical use of PIs is that patients who fail PI-containing regimens often have virus that lacks protease mutations, in apparent violation of fundamental evolutionary theory. Here we show that these unresolved issues are related and can be explained through analysis of the effects of PIs on distinct steps in the life cycle. PIs do not affect virion release from infected cells but block entry, reverse transcription (RT), and post-RT steps. The overall dose-response curves can be reconstructed by combining the curves for each step using the Bliss independence principle. Thus independent inhibition of multiple distinct steps in the life cycle generates the highly cooperative dose-response curves that make these drugs uniquely effective. Approximately half of the inhibitory potential of PIs is manifest at the entry step, likely reflecting interactions between the uncleaved Gag and the cytoplasmic tail (CT) of the Env protein. Sequence changes in the CT alone, which are ignored in current clinical tests for PI resistance, can confer PI resistance, providing an explanation for PI failure without resistance.

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INTRODUCTION

HIV-1 protease inhibitors (PIs) have played a critical role in the success of highly active antiretroviral therapy (HAART) (1-3). PIs are the key drugs in two of the four recommended initial HAART regimens and are also extremely important in salvage therapy for patients who fail initial regimens (1). Among all HIV-1 drugs, PIs have the highest intrinsic antiviral activity (2,4). PIs are the only antiretroviral drugs that have been successfully used in monotherapy (5). The high antiviral activity of this class results in large part from steep, highly cooperative dose-response curves (2,4), the molecular basis of which is not fully understood (6).

The PIs are substrate or transition state analogues that inhibit the activity of HIV-1 protease. This enzyme cleaves viral polyproteins during virus maturation (7). Among the protein products of the HIV-1 genome are three polyproteins: the envelope (Env) precursor protein (gp160), the Gag precursor protein (Pr55Gag), and Gag-Pol precursor protein (Pr160Gag-Pol). A cellular protease cleaves gp160 into the surface and transmembrane subunits, gp120 and gp41, respectively (8). These subunits remain associated, and trimers of gp120/gp41 complexes constitute the surface spikes that mediate viral entry. In contrast, the Gag and Gag-Pol polyproteins are each cleaved into multiple mature virion proteins by HIV-1 protease. The cleavages carried out by HIV-1 protease occur within the nascent virus particle and produce mature virions capable of infecting new cells.
While the interaction of PIs with the target enzyme is well understood at the structural and biochemical level (9-12), it remains unclear where in the virus life cycle the inhibition of virus maturation becomes manifest. Virus maturation is generally considered to be important for early post-entry steps including uncoating and reverse transcription (13-15). Both the reverse transcriptase (RT) and integrase enzymes are generated from Pr160Gag-Pol by cleavages carried out by HIV-1 protease. However, inhibition of the proteolytic cleavages necessary for maturation could in principle affect other steps as well. Studies of mutant viruses incapable of completing the necessary proteolytic cleavages suggest that immature particles are defective in entry (16-18). Interactions between the cytoplasmic tail (CT) of gp41 and uncleaved Pr55Gag appear to inhibit the fusion of immature particles. Despite the importance of PIs in HIV-1 treatment, the precise step(s) in the virus life cycle blocked by these drugs under clinical conditions has not been clearly defined.

Understanding where the PIs act in the virus life cycle is also important for understanding the resistance that arises in some patients on PI-based regimens. Resistance to PIs can occur through mutations in the protease gene (19), but the majority of patients failing PI-containing regimens do so without mutations in protease (20-23). This interesting and unexpected observation appears to violate the basic evolutionary tenets that govern other types of HIV-1 drug resistance. It also poses an important clinical dilemma – should treatment be changed for patients with detectable viremia but no mutations in protease? One possibility is that standard assays for resistance ignore parts of the HIV-1 genome that may contain mutations conferring resistance to PIs.
To understand the molecular mechanisms responsible for the high antiviral activity of PIs and the unusual features of resistance to these drugs, we carried out a detailed dissection of various steps in the viral life cycle affected by inhibition of protease in Chapter 1. We experimentally isolated each relevant step of the life cycle and measured the dose-response curves of PIs for each isolated step. We then reconstructed the overall dose-response curve by combining the curves from each step. This analysis provided a mechanistic explanation for the unique pharmacodynamics and exceptional efficacy of PIs. It also provided a potential explanation for PI resistance in the absence of mutations in protease. In chapter 2, we showed that the most commonly used clinical to detect phenotypic resistance is unable to capture the entire does-response curve for PIs. In chapter 3, we demonstrate that mutations in the viral envelope protein are sufficient to confer resistance to the PI. We isolated envelope proteins from patients on HAART and demonstrated that these envelope proteins contain PI-resistance mutations. In the light of these findings, a reevaluation of current clinical assays which detect resistance to protease inhibitors is necessary.
CHAPTER 1: THE STEEP DOSE-RESPONSE CURVES FOR PIS COULD BE EXPLAINED BY THEIR BLOCKAGE OF MULTIPLE STEPS IN THE VIRAL LIFE CYCLE.
Using single round infectivity assays, we have previously demonstrated unexpected complexity in the dose-response curves for antiretroviral drugs (2,4). At least two drug specific parameters are necessary to fully describe these curves: the concentration of drug producing 50% inhibition ($IC_{50}$) and the slope ($m$), which describes the steepness of the curve. The fraction of infection events unaffected ($f_u$) by drug at a concentration $D$ is given by the median effect equation (24):

$$f_u = \frac{1}{1 + \left(\frac{D}{IC_{50}}\right)^m}$$

Equation 1

or

$$\log\left(\frac{1 - f_u}{f_u}\right) = m \log\left(\frac{D}{IC_{50}}\right)$$

Equation 2

Standard semi-log dose response curves plot $f_u$ vs. log $D$. In comparing multiple drugs, it is useful to normalize the drug concentration by the $IC_{50}$. Plots of this kind obscure the importance of the slope parameter (Figure 1A). The dramatic effects of the slope parameter are much more evident in log-log plots or plots based on the median effect equation (Equation 2). Using this equation, dose-response curves that follow the standard Hill or sigmoidal $E_{max}$ models can be linearized. The $m$ values can be obtained directly from the slopes of the resulting lines. Generally, $m$ values greater than 1 reflect cooperative interactions (6,25,26). The clinical significance of the slope lies in the fact that with high slope values, small increases in drug concentration result in large increases in inhibition as shown in log-log and median effect plots (Figure 1A). In this context, the dose-response curves of PIs have two important features: first, the curves are steep, with an overall slope >1. Second, the median effect plots are non-linear, with a
pronounced upward inflection (Figure 1A, median effect plot). This upward inflection is not explained by any standard pharmacodynamic model. As a consequence of these two features, small increases in PI concentration can result in dramatically increased inhibition.

We hypothesized that these unique features of PI dose-response curves reflect the fact that virus particles generated in the presence of PIs can be blocked at multiple downstream steps in the virus life cycle (Figure 1B). Generally, failure to complete a step in the virus life cycle precludes all subsequent steps. Although PIs block the maturation of the virus particle, the subsequent step in the life cycle at which the failure in maturation becomes manifest is not completely understood, and it is possible that multiple downstream steps are affected (Figure 1B,C). Viruses that successfully complete the first downstream step (step A) could still be blocked at a subsequent step. The inhibition at the second step (step B) would be independent because only viruses that were not inhibited at step A can be blocked at step B. In this situation, the Bliss independence model can be used to compute the combined effects (27). As shown in Figure 1C, if $f_{uA}$, $f_{uB}$, and $f_{uC}$ are the fractions of viruses that are blocked at steps A, B, and C of life cycle, then the fraction of viruses that can complete the entire life cycle ($f_u$) is:

$$f_u = f_{uA} \times f_{uB} \times f_{uC}$$

Equation 3

Each inhibited step has its own dose-response curve, which can be described with $IC_{50}$ and $m$ values. Thus,

$$f_u = \frac{1}{1 + \left(\frac{D}{IC_{50_A}}\right)^m_A} \times \frac{1}{1 + \left(\frac{D}{IC_{50_B}}\right)^m_B} \times \frac{1}{1 + \left(\frac{D}{IC_{50_C}}\right)^m_C}$$

Equation 4
This equation predicts steep dose-response curves that inflect upward to give a maximal slope that can be shown to be equal to the sum of the slopes of the dose-response curves at steps A, B, and C (Appendix 1). Thus the steep dose-response curves for PIs could be explained by the fact that a single drug blocks multiple downstream steps in the life cycle.

To test this hypothesis, we developed experimental methods to isolate each potential step at which virions generated in the presence of PIs could be blocked. Using these assays, we measured the $IC_{50}$ and $m$ for each step of the life cycle inhibited by PIs and reconstructed the overall dose-response curves using the Bliss independence model (4,27).
Figure 1: Pharmacodynamics of PIs.

(A) Different representations of the dose-response curves for inhibition of HIV-1 infectivity by the PI atazanavir (ATV). Viruses generated in the presence of various concentrations of ATV were used to infect primary CD4+ T cells in a single round infection, and the fraction in infection events unaffected by drug (\(f_u\)) was measured as previously described (2). The left panel shows a conventional semi-log dose response curve in which \(f_u\) is plotted against the log of the drug concentration (normalized by the IC\(_{50}\)). Conventional plots obscure the differences between the ATV curve and the curve for a hypothetical drug with the same IC\(_{50}\) and an \(m\) value of 1 (dotted line). The middle panel shows a log-log dose response curve which better illustrates that ATV produces much more inhibition at high drug concentrations than a hypothetical drug with the same IC\(_{50}\) and an \(m\) value of 1. The right panel shows a median effect plot, log \([1-f_u]/f_u\) vs. log \(D/IC_{50}\). This plot, which is based on Equation 2, linearizes most dose-response curves, giving lines whose slope values are equal to the slope parameter or Hill coefficient (\(m\) in Equations 1 and 2). This plot illustrates the steep slope and upward inflection of the PI dose response curve. The dotted line shows the expected curve for a drug with the same IC\(_{50}\) and an \(m\) value of 1. (B) PIs could inhibit multiple steps in the life cycle. PIs block the cleavages necessary for maturation of the virus particle (green arrow). Since these cleavages take place simultaneously with budding, an effect on budding is possible. Viruses that fail to mature due to the action of PIs could be blocked at multiple downstream steps, including entry, reverse transcription (RT), and integration. (C) If PIs block multiple downstream steps, then by the Bliss independence model, the fraction of successful infection events is the product of the fraction of viruses that pass each block. The maximal slope of the overall dose-response curve approaches the sum of the slopes of the dose response curves for inhibition of each step (Appendix 1).
CHAPTER 1.1: PIS DO NOT BLOCK BUDDING
The processes of viral budding and maturation occur concomitantly (28). Although the Gag polyprotein is necessary and sufficient for budding (29-31), it is conceivable that PIs could affect this step. For example, several PIs inhibit proteasome function (32-34) and some proteasome inhibitors, such as Epoxomicin, block retroviral budding (35,36).

To determine whether inhibition of virion maturation affects budding, we developed a novel assay for the detection of virus particle released from virus-producing cells. Synchronous analysis of the budding step can be carried out using cells transfected with proviral constructs. However, in this situation, analysis of virus particle release by RT-PCR is complicated by the presence of plasmid DNA even after DNase treatment. Therefore, we developed an RT-PCR method that allowed specific quantitation of genomic viral RNA in virions released from transfected cells even in presence of micromolar concentrations of contaminating HIV-1 DNA. 293T cells were co-transfected with a plasmid carrying the HIV-1 NL4-3 provirus with green fluorescence protein (GFP) in the env open reading frame (ORF) (NL4-3ΔEnv) and a vector expressing an X4-tropic Env (from NL4-3). After 48 hours of incubation in the presence of PIs, supernatants were treated with a genetically modified bacterial endonuclease (Benzonase) that degrades all forms of DNA and RNA including single and double stranded forms and circular and linear forms. This enzyme can thus degrade free, extravirion RNA, plasmid DNA, and proviral DNA. Intra-virion viral RNA was then measured by quantitative RT-PCR. The 3’ primer consisted of 25 dTs followed by 5 nucleotides (GAAGC) complementary to the last 5 nucleotides in the R region of the LTR. This primer hybridizes specifically to HIV-1 mRNAs. The 5’ primer anneals to a highly conserved region in the U3 region LTR. Unlike other viral quantification assays
that use RT-PCR, this assay is unaffected by contaminating proviral DNA or plasmid DNA since only polyadenylated mRNA can be detected (Figure 2). In addition, pretreatment of supernatant with a nuclease ensures that only intra-virion RNA will be measured and not mRNA released from dead cells (Figure 3). Unlike an ELISA-based assay that quantitates the amount of HIV-1 Gag p24 antigen in the supernatant, the RT-qPCR based assay described here is independent of the maturation state of the virion and detects only particles containing viral RNA.
Figure 2: An assay for virus production that is not affected by plasmid DNA.

293T cells were co-transfected with a plasmid carrying the NL4-3 provirus with GFP in the env ORF (NL4-3ΔEnv) and a vector expressing an X4-tropic envelope. At 48 hours post-transfection, viral RNA in the supernatant is measured by RT-qPCR. The 3’ primer consisted of 25 dTs followed by 5 nucleotides (GAAGC) complementary to the last 5 nucleotides in the R region of the LTR. This primer hybridizes specifically to HIV-1 mRNAs (Shan et al., J.Virol. 2013). The 5’ primer anneals to a highly conserved region in the U3 region LTR. In control tubes in which reverse transcriptase was omitted, the signal detected was not significantly above the background. Epoxomicin is a proteasome inhibitor and has been shown to interfere with viral assembly and budding (30,31). Epoxomicin caused a 100-fold reduction in virus release. The assay sensitivity is 10 copies.
Figure 3: Nuclease treatment prior to viral RNA extraction allows specific detection of intra-virion HIV-1 RNA.

293T cells were co-transfected with a plasmid carrying the NL4-3 provirus with GFP in the env ORF (NL4-3ΔEnv) and a vector expressing an X4-tropic envelope. At 48 hours post-transfection, the supernatant was treated with 1 unit/200μl of nuclease either before or after lysis of viral membranes with Triton X-100. After 10 minutes incubation in 37°C, a second lysis buffer (AVL from Qiagen) was added prior to RNA isolation. This second lysis buffer is highly denaturing which causes inactivation of the nuclease enzyme as well as lysis of the viral membrane in samples that were not previously lysed by the triton X-100. Viral RNA in the supernatant is measured by RT-qPCR using primer pairs described above. In control experiments, a viral construct with a stop codon in the reading frame of Gag (NL4-3ΔGag) was used. In the absence of Gag, virus particles do not assemble and bud from the producing cells, the signal detected in these samples is not significantly above the level of background. The assay sensitivity is 10 copies. ND, none detected.
Using this assay, we demonstrated that virion release from virus-producing cells is not affected by PIs. Figure 4 shows results for the three most widely used PIs: atazanavir (ATV), darunavir (DRV), and lopinavir (LPV). The log-log dose response curves show no decrease in the release of virus particles as the PI concentration increases. This is true even at concentrations up to 100 fold above the IC₅₀ for inhibition of infectivity and even for PIs reported to block the cellular proteosomal activity (32-34) (Figure 5).
Figure 4: Log-log dose response curves illustrating the effects of the PIs ATV, DRV, and LPV on budding (A,C,E) and entry (B,D,F).

Budding was assessed quantifying virus particles in the supernatants of cultures of 293T cells transfected with a proviral construct. Entry was measured by FRET using BLAM-vpr-loaded pseudoviruses with wild type HIV-1 Env (closed circles), HIV-1 Env with a truncated CT (open circles), or VSV-G (open triangles). Drug concentrations are normalized by previously measured IC$_{50}$ values for inhibition of infectivity by each drug (13.6 nM, 23.6 nM, and 35.8 nM for ATV, DRV and LPV, respectively, reference 2).
Figure 5: PI treatment of virus-producing cells does not inhibit viral budding

293T cells were co-transfected with NL4-3ΔEnv-GFP and a vector expressing an X4-tropic Env. Cells were then distributed in 96-well plates, and PIs were added. At 48 hours after transfection, supernatants were treated with 1 unit/150μl of nuclease to degrade free, extra-virion RNA. Viral RNA in the supernatant was measured by quantitative RT-PCR using primer-probe pair specific for HIV-1 mRNA. IC50 values refer to the overall PI IC50 measured in (2) and are 144.2 nM, 166.8 nM, and 90.9 nM for APV, NFV and IDV, respectively.
CHAPTER 1.2: VIRUSES PRODUCED IN THE PRESENCE OF PIs SHOW
DEFECTS IN ENTRY
To determine whether PIs inhibited entry, we utilized a previously described assay for viral entry based on fluorescence resonance energy transfer (FRET) \(^{(37)}\). Briefly, the enzyme beta-lactamase was incorporated into virus particles as a fusion protein with the HIV-1 accessory protein Vpr \((\text{BLAM-}Vpr)\). Upon entry, \text{BLAM-}Vpr cleaves a fluorescent dye that is preloaded into the target cells, resulting in a shift in the emission spectrum detectable with flow cytometry. Importantly, we first showed that PI treatment does not affect the activity of the \text{BLAM-}Vpr fusion protein \((\text{Figure 6})\).
Figure 6: The enzymatic activity of the BLAM-Vpr fusion protein is not dependant on cleavage by HIV-1 protease and is not affected by the action of PIs.

(A) BLAM-Vpr fusion protein has the same level of enzymatic activity as the beta-lactamase enzyme. 293T cells were transfected with either Blam-Vpr or a beta-lactamase-encoding construct. At 24 hours post-transfection, the cells were incubated with the substrate CCF2-AM. The enzymatic activity was quantified as the ratio of blue to green color change by flow cytometry. (B) Western Blot analysis to assess expression of BLAM-Vpr and beta-lactamase in the cells used in A. (C) The enzymatic activity of BLAM-Vpr is not dependent on the action of HIV-1 protease and is not affected by the action of PIs. 293T cells were transfected with Blam-Vpr alone or along with the NL4-3ΔEnvGFP construct expressing HIV-1 protease in the presence or absence of 1 μM LPV. At 24 hours post-transfection, the cells were incubated with the substrate CCF2-AM. The enzymatic activity was quantified as the ratio of blue to green color change by flow cytometry. (D) Cells transfected with the NL4-3ΔEnvGFP construct express functional HIV-1 protease. 293T cells transfected with the NL4-3ΔEnvGFP were washed three times in cold PBS 24 hours after transfection. Cell lysates were analyzed via Western Blot for the presence of fully processed Capsid protein using an anti-p24 antibody.
To assess the effects of PIs on HIV-1 entry, HEK 293T cells were co-transfected with NL4-3ΔEnv, an expression vector for an X4-tropic Env, and a plasmid expressing BLAM-Vpr. Transfected cells were then distributed into 96-well plates, and PIs were added. After 48 hours of incubation in the presence of PIs, virus-containing supernatants were collected and used to infect primary CD4+ T lymphoblasts. Entry was assessed by flow cytometry as a green→blue shift in individual cells as described (37).

All PIs tested produced a dose-dependent inhibition of the entry of pseudoviruses carrying wild type HIV-1 Env. As shown in the log-log dose response curves in Figure 4, the fraction of entry events unaffected by drug (f_u) decreased with increasing drug concentration for ATV, DRV, and LPV. This entry inhibition was dependent on the nature of the envelope protein used. Pseudoviruses generated using the vesicular stomatitis virus G protein (VSV-G) rather than HIV-1 Env were not inhibited at entry by PIs. In addition, truncation of the gp41 CT largely relieved the PI-mediated inhibition of entry (Figure 4). These results are consistent with previous studies showing that viruses with mutations in the Gag cleavage sites recover their ability to fuse if pseudotyped with VSV-G or an HIV-1 envelope that has a truncation of the gp41 CT (17,18). In these viruses, interactions between the gp41 CT and the matrix (MA) component of uncleaved Gag precursor protein (Pr55Gag) that may inhibit entry cannot take place. In subsequent experiments, we used virus particles pseudotyped with VSV-G or with an HIV-1 Env with a truncated gp41 CT to bypass the entry inhibition of PIs and examine the effects of these drugs on subsequent steps in the life cycle.
CHAPTER 1.3: VIRUSES PRODUCED IN THE PRESENCE OF PIS ARE
BLOCKED AT MULTIPLE STEPS IN THE LIFE CYCLE.
Because the BLAM-Vpr assay detects only effects on the entry step, the above results clearly demonstrate that PIs inhibit HIV-1 entry. To compare the effects of PIs on different steps in the life cycle, we used median effect plots (Figure 7). Unlike the upwardly inflected dose-response curves for the overall inhibition of infectivity (Figure 1A, median effect plot, and references 2 and 4), the inhibition of entry resulting from PI treatment of virus-producing cells gave linear dose response curves throughout the dynamic range of the assay (up to 1.5 logs above the $IC_{50}$). By extrapolating these straight lines into the clinical concentration range, we estimated that at peak plasma concentrations ($C_{max}$), ATV, DRV, and LPV produce 2.8, 5.0 and 2.7 logs of inhibition at the entry step, respectively.

The inhibition of entry by PIs is not complete. For some virions generated in the presence of PIs, entry occurs and downstream steps in the viral life cycle can take place. To evaluate the effect of PIs on reverse transcription, we infected CD4$^+$ T lymphoblasts with HIV-1 pseudoviruses carrying a truncated form of gp41. As shown in Figure 4, PIs have only a minimal inhibitory effect on the entry of viruses with a truncated gp41 CT at concentrations up to 10 fold above the $IC_{50}$. This allowed us to isolate post-entry effects. Pseudoviruses were made by co-transfecting 293T cells with NL4-3ΔEnv and an expression vector for an X4-tropic Env truncated at L753 of the gp41 CT (HXB2 coordinates). At 48 hours after transfection, virus-containing supernatants were collected and used to infect primary CD4$^+$ T cells from healthy donors. At 36 hours after infection, CD4$^+$ T cells were washed and treated with the broad specificity endonuclease Benzonase to remove residual plasmid DNA. After three washes in cold phosphate buffered solution (PBS), the cells were lysed, and DNA was isolated for quantitation of early, intermediate,
and late reverse transcripts by qPCR (38). The primers used to detect early reverse transcription products also detect intermediate and late products, and thus measurement of early products can be used to determine the overall production of proviral DNA in recently infected cells. The ability to wash cells thoroughly in cold PBS in addition to the nuclease treatment allowed us to remove most, if not all, of the plasmid DNA contamination. This is in contrast to the above situation in which virus particles are not easily separable from contaminating plasmid DNA. In control experiments, we showed that the detection of reverse transcripts was largely blocked by addition of the fusion inhibitor enfuvirtide at the time of infection. In addition, no reverse transcription products were detected when a pseudovirus with an inactivating mutation in RT was used (Figure 8). These results demonstrate that the assay detects newly synthesized viral DNA in recently infected cells.

Using this approach, we determined whether commonly used PIs affect reverse transcription. Figures 7B, F and J show dose-response curves for PI-mediated inhibition of reverse transcription. To varying degrees, all PIs tested inhibited the appearance of reverse transcripts in a system where the entry block is largely bypassed.

Following reverse transcription, the nascent viral DNA is integrated into cellular DNA, a process catalyzed by the viral enzyme integrase (IN). IN is produced by protease-mediated cleavage of the Pr160Gag-Pol precursor protein. Therefore, PIs can in principle also inhibit integration by preventing the formation of functional IN. Due to the heterogeneous nature of HIV-1 integration sites within the human genome (39,40), available assays for integration, such as Alu-PCR-based assays (41-43), lack the precision required for analyzing the dose-response curves of PIs at the integration step.
Instead, we utilized VSV-G pseudotyped viruses, which are inhibited only at post-entry steps (Figure 4), to quantify the combined inhibition by PIs of all post-entry steps, including integration (Figure 7). For all PIs tested, dose response curves for the combined inhibition of all post-entry steps were significantly steeper than the curves for inhibition of reverse transcription (P=0.0086, 0.00066, and 0.022 for ATV, DRV, and LPV, respectively), indicating that inhibition at a post-reverse transcription step is also contributing to the overall inhibition (Figure 7). Interestingly, dose response curves for the combined inhibition at all post-entry steps also showed lower IC$_{50}$ values than the IC$_{50}$ values for the inhibition of entry. Taken together, these results demonstrate that PIs affect multiple distinct steps in the life cycle including both entry and post-entry events.
Figure 7: Median effect plots illustrating the effects of the PIs ATV, DRV, and LPV on HIV-1 entry, reverse transcription, all-post entry events, and overall infectivity.

(A,E,I) Effect of PIs on viral entry. The dose-response curves of PIs at the entry step from Figure 4 were linearized by plotting \( \log\left(\frac{(1-f_i)f_u}{f_i}\right) \) vs. \( \log\left(\frac{D}{IC_{50}}\right) \).

(B,F,J) Effect of PIs on reverse transcription. RT-qPCR was used to measure production of early reverse transcripts in primary CD4+ T lymphoblasts infected with pseudoviruses carrying an X4-tropic Env truncated in the cytoplasmic tail of gp41 CT. PIs were present at the indicated concentration during virus production.

(C,G,K) Effect of PIs on all post-entry steps. Flow cytometry was used to detect infection of primary CD4+ T lymphoblasts by pseudoviruses carrying a VSV-G. PIs were present at the indicated concentration during virus production.

(D,H,L) Reconstruction of overall dose-response curve of PIs by combining the dose-response curves at entry and post-entry steps. A two-step form of Equation 3 was used to combine best fit dose response curves for PI effects on entry (blue line) and all post-entry steps (red line). The resulting curves (dotted black lines) were compared to experimental result for the inhibition of infectivity by PIs (black circles).
Figure 8: Quantitation of HIV-1 early, middle, and late reverse-transcription products in primary CD4+ T lymphoblasts 36 hours after infection.

293T cells were co-transfected with NL4-3ΔEnv and an X4-tropic envelope. Supernatants were collected at 48 hours and used to infect primary CD4+ T lymphoblasts. At 36 hours after infection, CD4+ T cells were washed and treated with nuclease to remove residual plasmid DNA. Cells were then washed 3x with cold PBS to remove the nuclease. Cells were then lysed, and DNA was isolated. Early, middle, and late RT products were quantified by qPCR (38). Treatment of cells with the fusion inhibitor enfuvirtide prior to the addition of virus prevented the appearance of RT products as did the use of virus with an inactivating D185N mutation in the active site of RT.
CHAPTER 1.4: RECONSTRUCTING PI DOSE-RESPONSE CURVES BASED ON INHIBITION AT INDIVIDUAL STEPS OF VIRAL LIFE CYCLE
Having separately measured inhibition by PIs at entry and post entry steps, we determined whether the overall dose-response curves of PIs could be predicted by assuming that the fraction of successful infection events is the product of fraction of events unaffected at each of the relevant steps in the life cycle (Figure 1B, 1C and Figure 9). This is the same assumption in the Bliss model of drug-drug interaction that is used to predict the overall inhibition achieved by two drugs inhibiting different step in the life cycle (4,27). Using the Bliss model to combine entry and post-entry inhibition by the PIs, we successfully predicted the overall dose-response curves for all PIs tested. Results for the most commonly used PIs (ATV, DRV, LPV) are shown in Figure 7. The theoretical curves matched the experimentally obtained dose-response curves with $R^2$ values of 0.97, 0.94 and 0.97 for ATV, DRV and LPV, respectively. The upward inflection of the overall dose response curves reflects that fact that at higher concentrations, infection is blocked at the entry step as well as at post-entry steps. Thus, the unique pharmacodynamic features of PI dose-response curves, i.e. the steepness and concave shape, can be explained by the fact that viruses generated in the presence of the PIs are inhibited at multiple steps in the life cycle.
Figure 9: The overall dose-response curve of a drug acting at two different steps of life cycle.

(A) A hypothetical drug blocking two different steps of life cycle, A and B. Assuming that the inhibition at each step is independent of the previous step(s), Bliss model of drug-drug interaction (47) can be used to predict the overall fraction of viruses that can complete the life cycle ($f_u$) from the fraction of viruses that complete each individual step. (B) The overall median effect dose-response curve of a drug acting at two subsequent steps of life cycle inflects concaves up to approximate the dose-response curve of a hypothetical drug with slope equal to the sum of the slopes at each individual step. The blue and purple curves are dose-response curves for the inhibition of steps A, and B, respectively. The solid black curve is the overall dose-response curve, and the dotted black curve is the dose-response curve of a hypothetical drug with slope equal to the slopes at steps A and B. ($IC_{50A}$, $IC_{50B}$, $m_A$ and $m_B$ used here are: 214 nM, 33 nM, 2, and 1, respectively).
We also examined the dose-response curve for PI-mediated inhibition of the generation of the Gag subunit p24, which requires multiple cleavages of the Gag precursor protein during virus maturation (Figure 10). The dose-response curve for inhibition of this process is steep \((m=2.0)\), likely reflecting the participation of multiple copies of HIV-1 protease in the maturation of each virion (6). There is no upward inflection because this curve reflects a biochemical process and not the combined effects of inhibition at multiple independent steps in the life cycle.
Figure 10: Dose-response curve for PI-mediated inhibition of processing of Pr55Gag.

HEK 293T cells were co-transfected with an NL4-3ΔEnv vector expressing wild type gag and pol and with vectors expressing X4-tropic HIV-1 env. ATV was added to the cells at the indicated concentrations. Two days later, supernatants were analyzed using Western Blotting for p24 in triplicate. (A) A representative blot. (B) The intensities of the p24 bands were quantified using ImageJ software and the results plotted according to the median-effect model (Equation 2). Drug concentrations are normalized by previously measured IC50 value for inhibition of infectivity by ATV (13.6 nM, reference 2).
CHAPTER 1.5: AT CLINICAL CONCENTRATIONS, THE ENTRY INHIBITION BY PIS IS A MAJOR COMPONENT OF THEIR OVERALL INHIBITORY POTENTIAL
Since the median effect plots of the dose response curves of PIs at individual steps of life cycle are linear (Figure 7), we extrapolated these plots to estimate the degree of inhibition of each individual step of life cycle at the peak plasma concentration of each drug ($C_{\text{max}}$). Inhibition at post-RT steps was determined by assuming that the post-entry inhibition results from independent inhibition at reverse transcription and post-reverse transcription steps. Since the inhibition at reverse transcription and the combined inhibition at all post-entry steps were measured directly (Figure 7), we used the Bliss independence model to obtain the inhibition at post-reverse transcription steps. Figure 4 shows the fraction of the overall inhibition that occurs at each step in the life cycle for commonly used PIs at their peak plasma concentration, $C_{\text{max}}$. DRV causes 9.2 logs of inhibition of new infection events at $C_{\text{max}}$, 4.2 logs of which is due to inhibition of entry and 2.0 logs of which is due to inhibition of reverse transcription. The remainder reflects inhibition of post-reverse transcription steps. A similar breakdown was observed for the other commonly used PIs. For each PI tested, inhibition at the entry step was most prominent, with inhibition at the RT and post-RT steps accounting for smaller and variable fractions of the total inhibition. These results demonstrate the importance of the entry inhibition in the overall effect of PIs.

Because the effects of PIs on entry are evident at higher drug concentrations, the relative contributions of inhibition at entry and post-entry steps to the overall inhibitory effect of PIs vary with drug concentration. It is expected that entry inhibition will contribute to antiviral effect in vivo throughout the interval between doses, as drug concentrations are not expected to decline to levels lacking this effect. However, drug concentrations may decrease to levels that have a decreased anti-entry effect during
prolonged periods (2-4 days) of poor adherence. Therefore, with poor adherence, anti-
entry effects may make a smaller relative contribution to the overall inhibition mediated
by PIs.
Figure 11: Contribution of the inhibitory effect of PIs on each step of viral life cycle to the overall inhibitory effect at $C_{\text{max}}$.

The linear dose-response curves of PIs at entry, reverse transcription and post-reverse transcription steps were extrapolated to predict the inhibition of each step at $C_{\text{max}}$. 
Due to the significant contribution of entry inhibition to the overall inhibition produced by PIs (Figure 11), we hypothesized that drug resistance mutations would alter dose-response curves for both the entry and post-entry events. To test this hypothesis, we introduced two LPV resistance mutations, V82A and V82F (15) into the NL4-3ΔEnv provirus by site directed mutagenesis to create NL4-3ΔEnvV82A and NL4-3ΔEnvV82F, respectively. 293T cells were co-transfected with NL4-3ΔEnvV82A or NL4-3ΔEnvV82F, along with either a VSV-G expression vector or an HIV-1 Env expression vector and the BLAM-Vpr construct. HIV-1 Env-pseudotyped particles incorporating the BLAM-Vpr fusion protein were used to study the effect of protease resistance mutations on the inhibition of HIV-1 entry by LPV (37), and the VSV-G pseudotyped particles were used to study the inhibition at post-entry steps using a single-round infectivity assay with GFP expression as the readout (44). The results are shown in Figures 12A and 12B. These mutations produced resistance by altering both entry and post-entry dose response curves. However, as shown in the next section, one of the two commercial phenotypic assays now in widespread clinical use is not capable of accurately measuring the effect of PIs on entry because an HIV-1 Env is not used.
Figure 12: Effect of PI-resistance mutations in the protease gene on inhibition of entry and post-entry steps of viral life cycle.

(A) Effect of LPV-resistance mutations in the protease gene on HIV-1 entry. 293T cells were co-transfected with NL4-3Δ Env vector expressing either wild type protease (WT) or one expressing the protease mutations V82A or V82F, a vector expressing an X4-tropic HIV-1 envelope, and Blam-Vpr. Viruses were produced in the presence of increasing concentrations of LPV, and a highly sensitive FRET-based entry assay was then used to quantitate the amount of entry into primary CD4+ T cells. (B) Effect of PI-resistance mutations in the protease gene on post-entry events. 293T cells were co-transfected with the NL4-3ΔEnv vector expressing either wild type protease (WT) or one expressing the protease mutations V82A or V82F, and a vector expressing VSV-G. The transfected cells were then plated in 96-well plates and LPV was added. Two days after the transfection, the viral supernatant was used to infect CD4+ lymphoblasts. Three days after infection, GFP-expressing cells were quantified using flow cytometry.
CHAPTER 2: IMPORTANCE OF THE HIV-1 ENV PROTEIN IN
THE MEASUREMENT OF RESISTANCE TO PIs
Genotypic and phenotypic assays for drug resistance play an important role in the management of HIV-1 infection. In the most commonly used phenotypic assay (45), a 1.5-kb region of the pol gene spanning the p7-p1-p6 protease cleavage sites in Gag, protease, and a portion of the RT coding region is cloned into an indicator HIV-1 vector expressing luciferase. Pseudoviruses are made in 293T cells by co-transfecting this vector with an expression vector encoding an amphotropic murine leukemia virus envelope protein (MLV-E). The resulting pseudoviruses are then used to infect fresh 293T cells. Dose-response curves for the inhibition of infection by pseudoviruses carrying patient-derived pol sequences are compared to curves for a wild type reference strain. A shift in the IC\textsubscript{50} greater than an empirically determined value is reported as evidence of phenotypic resistance (45).

The transmembrane subunit of the MLV-E is a 15-kDa protein (p15) that is cleaved in the cytoplasmic domain by the MLV protease into a 12-kDa protein (p12) and a 16-residue peptide (p2). This cleavage activates the fusion potential of MLV-E. When Env-defective HIV-1 proviruses are pseudotyped with MLV-E, the HIV-1 protease cleaves p15 and renders the envelope fusogenic (12,46). We hypothesized that PIs might inhibit the entry of MLV-E-pseudotyped particles by preventing this essential cleavage. Such an inhibitory effect on a clinically irrelevant substrate, the MLV-E, could artificially alter the dose-response curves of the PIs and compromise the analysis of resistance.

To evaluate this hypothesis, we tested the effect of HIV-1 protease enzyme function on entry of HIV-1 pseudoviruses expressing either the wild type MLV-E (p15) or a mutant form of MLV-E (p12) with a stop codon at the protease cleavage site. To generate these pseudoviruses, p15 or p12 expression vectors were co-transfected into 293
T cells along with the BLAM-\textit{Vpr} vector and the NL4-3\textDelta Env proviral construct encoding either wild type protease or protease with an inactivating active site mutation (D25N). After normalization based on viral RNA copy number, the resulting pseudoviruses were used to infect activated primary CD4\textsuperscript{+} cells, and entry was measured using the FRET-based fusion assay. Figure 13A shows that functional HIV-1 protease is essential for entry of pseudoviruses with MLV-E since protease-defective pseudoviruses are unable to enter. This entry block is alleviated by truncating the MLV-E cytoplasmic tail at the site of protease cleavage (Figure 13A), consistent with previous studies (46).

To determine whether HIV-1 PIs inhibit the entry of MLV-E-pseudoviruses, we transfected 293T cells with expression vectors for MLV-E and BLAM-\textit{Vpr}, and with the NL4-3\textDelta Env proviral construct in presence of the PI DRV. After 48 hrs, supernatants containing newly generated virus particles were used to infect activated primary CD4\textsuperscript{+} T cells. Figure 13B shows that DRV inhibits the entry of pseudoviruses carrying WT MLV-E (p15). In contrast, entry mediated by the truncated form of MLV-E (p12) was only weakly inhibited by DRV. Clinical assays to detect protease resistance use pseudoviruses containing MLV-E. To investigate the extent to which the use of MLV-E-affects the overall dose-response curves of PIs, we examined DRV-mediated inhibition of the infection of 293T cells or primary CD4\textsuperscript{+} T cells by recombinant HIV-1 viruses carrying MLV-E or HIV-1 Env. The readout was the percent of GFP-positive cells at 2 days after infection. As shown in Figure 13C, dose response curves for inhibition of infection of CD4\textsuperscript{+} T cells by MLV-E pseudoviruses were steeper than the curves for inhibition of entry (Figure 13B). This likely reflects the fact that infectivity assays capture both entry and post-entry events. Truncation of the cytoplasmic tail had a smaller
effect on the dose response curve for infectivity than on the dose response curve for entry (compare Figures 13B and 13C). Again this is expected because the infectivity assay captures effects of the inhibition of protease function on multiple steps including entry, reverse transcription, and post-reverse transcription steps. Cell type differences are also apparent in the dose response curves (Figure 13D), with inhibition of infection of 293T cells by MLV-E pseudoviruses occurring at lower DRV concentrations than for inhibition of infection of CD4+ T cells. Finally, we compared the dose-response curves for inhibition of MLV-E pseudovirus infection of 293T cells and for inhibition of HIV-1 Env pseudovirus infection of primary CD4+ T cells (Figure 13E). The former represents the commercial resistance assay while the latter more closely reflects HIV-1 infection in vivo. The dose response curve for MLV-E pseudovirus infection of 293T cells is shifted to left by ~0.5 logs and does not show the upward inflection evident in dose-response curves for HIV-1 Env pseudovirus infection of primary CD4+ T cells. This may reflect the fact that the effect of protease on entry is different in the two cases. For MLV-E, the inhibited enzyme acts directly on the envelope protein. For HIV-1 Env, the protease acts on the Gag polyprotein which interacts with the cytoplasmic tail of Env. The net effect is that the shapes of the dose-response curves are different. These differences could influence the interpretation of clinical assays for drug resistance. At DRV concentrations around the IC50 (23.6 nM), the difference in inhibition is over 10 fold and is highly significant (P<0.015).
Figure 13: Importance of the entry effect on the analysis of resistance to PIs.

(A) Dependence of pseudoviruses with MLV-E on HIV-1 protease for entry. HIV-1 pseudoviruses with wild type or mutant (D25N) protease and the indicated forms of MLV-E were used to CD4+ lymphoblasts, and entry was measured by FRET. (B) Effect of PI-treatment of virus-producing cells on the entry of pseudoviruses with MLV-E. Pseudoviruses with the indicated forms of MLV-E and wild type protease were generated in the presence of increasing concentrations of DRV and tested for entry into primary CD4+ T lymphoblasts using FRET. (C) DRV-mediated inhibition of infection of CD4+ T lymphoblasts by HIV-1 pseudoviruses with wild type (p15) or truncated (p12) MLV-E. Infection was assessed by GFP-expression in target cells. (D) The effect of target cell type on PI dose-response curves. HIV-1 pseudoviruses with wild type MLV-E generated in the presence of increasing concentrations of DRV were used to infect 293T cells or primary CD4+ T lymphoblasts. Infection was assessed by GFP expression in target cells. (E) Comparison of DRV dose response curves in experimental systems representing the clinical assay for resistance (MLV-E/293T) or in vivo infection (HIV-1 Env/CD4). HIV-1 pseudoviruses with MLV-E or HIV-1 Env were generated in the presence of increasing concentrations of DRV and used to infect 293T cells or primary CD4+ T lymphoblasts. Infection was assessed by GFP expression in target cells.
CHAPTER 3: HIV-1 ENV MUTATIONS CAN CONFER PI RESISTANCE EVEN IN THE CONTEXT OF WILD TYPE GAG AND POL GENES.
PIs achieve their overall inhibition of viral replication by the combined effects of inhibition at multiple steps in the life cycle (Figure 7). Studies of Gag-Env interactions by others (17,18) have shown that interactions between the gp41 CT and the MA component of Pr55Gag have an inhibitory effect on fusion that is relieved by protease-mediated cleavage of Pr55Gag. We have shown (Figure 4) that treatment of virus-producing cells with PIs at clinical concentrations also inhibits viral fusion and that this inhibition contributes significantly to the overall inhibition produced by PIs (Figure 11). A single point mutation that causes premature termination of gp41 abolishes the inhibition of entry by PIs (Figure 4). In addition, resistance mutations in the protease gene alter both entry and post-entry dose-response curves (Figure 8). Therefore, we hypothesized that mutations in the \textit{env} gene that alter the interaction with MA might arise \textit{in vivo}. Such mutations could in principle confer some degree of PI resistance even in the context of wild type \textit{gag} and \textit{pol} genes and provide a selective advantage to the mutant viruses. Importantly, such viruses would be identified as drug-susceptible in both of the current clinical assays for resistance, since the \textit{env} gene is not included in genotypic analyses, and MLV-E is used in one of the common phenotypic assays.

To determine whether mutations in \textit{env} can confer PI resistance, we examined clinical isolates with high-level resistance to PIs. Koh \textit{et. al.} (47) isolated HIV-1 variants from a patient failing PI-containing regimens. These viruses contained 9 to 14 protease mutations associated with PI resistance. The viruses were then grown in the presence of DRV for 51 passages, and additional mutations in the protease gene accumulated (47). We cloned the full-length \textit{env} gene from passages 1 and 51 (E-1 and E-51). Pseudoviruses were made in 293T cells, in the presence of PIs, by co-transfecting the
cells with the NL4-3ΔEnv proviral construct and expression vectors for either the E-1 or E-51 Envs. Except for the \textit{env} gene, the HIV-1 genes in these constructs were wild type, including the \textit{pol} gene with the protease coding region and the \textit{gag} gene. Figures 14A, 14B, and 14C show that even in the context of wild type \textit{gag} and \textit{pol}, these mutant Envs affect the dose-response curves for PIs, flattening the curves and producing a substantial degree of resistance at PI concentrations 10 fold above the \textit{IC}_{50} for wild type virus. Interestingly, the flattening of dose-response curves is also characteristic of single resistance mutations in the protease gene (48). This effect was prominent for the PI DRV but was also observed for the other commonly used PIs, ATV and LPV. This resistance was more apparent at higher PI concentrations, consistent with the observation that the effect of PIs on entry requires higher drug concentrations than the effect on subsequent steps (Figure 7). At 132 nM (2 logs below \( C_{\text{max}} \) at a log \( D/IC_{50} \) value of 0.75 on the x-axis), the fraction of infection events blocked by DRV was 10 fold more when wild type Env was used to generate pseudoviruses compared to pseudoviruses with either the E-1 or E-51 envelopes.

Next, we studied patients on PI-based regimens who had detectable viremia. None of these patients had any major PI-resistance mutations as reported by standard clinical genotypic assays (Table 2). Full-length \textit{env} genes were cloned from the plasma or PBMC of these patients as indicated in Table 2. Pseudoviruses were made in 293T cells, in the presence of PIs, by co-transfecting the cells with the NL4-3ΔEnv proviral construct and expression vectors for patient-derived Envs. Except for the \textit{env} gene, the HIV-1 genes in these constructs were wild type, including the \textit{pol} gene with the protease coding region and the \textit{gag} gene. A total of 18 distinct functional \textit{env} clones from 6 different
patients were studied. Figures 14D, 14E, and 14F show that env genes cloned from two of these patients (PIE1 and PIE2) conferred significant PI resistance even in the context of wild type gag and pol genes. As in the example above, this resistance was apparent at 132 nM DRV which is well below the $C_{max}$ value of 15 µM (2).

Several different parameters were evaluated to assess the level of PI resistance conferred by patient-derived env sequences (Table 2). As indicated in Equation 1, antiviral activity is influenced by both the $IC_{50}$ and the dose response curve slope ($m$). Changes in either parameter can cause resistance to antiretroviral drugs, with decreases in $m$ being particularly important for PI resistance (48). Instantaneous inhibitory potential, IIP, is a parameter that takes into account both $IC_{50}$ and $m$. IIP is the number of logs by which a particular concentration of drug reduces single round infection (2). Resistance is manifest as a decrease in IIP. Table 2 shows that 9 of 18 patient-derived env clones conferred significant resistance as assessed by a statistically significant fractional decrease in IIP at $C_{max}$. Resistance mutations can decrease the replication capacity of the mutant virus in the absence of drug. Thus the actual selective advantage that a mutation confers in the presence of drug can be calculated by considering both the change in replication capacity and the degree of resistance (48). Selective advantage is ratio of the replication rate of mutant virus to the replication rate of the wild type virus under a given set of conditions. Table 2 also lists replication capacity and selective advantage for the patient-derived env clones. Some of the clones show a high selective advantage over wild type virus at high drug concentrations. Only 50% of the env clones analyzed conferred significant resistance, and no resistant clones were identified in samples from one patient (PIE4). Thus, Env sequences may contribute to PI failure only in a subset of
patients. Pseudoviruses generated with env genes cloned from a patient on a fully suppressive HAART regimen and a treatment-naïve chronic progressor did not confer any resistance to DRV (Table 2, Figure 15).

Since the gp41 CT is required for the inhibition of entry by PIs, we hypothesized that drug-resistance mutations in the env gene could be localized to this region in at least some cases. We created constructs expressing chimeric Env proteins with the CT of gp41 from the PI-resistant viruses E-1, E-51, PIE1, or PIE2, and the remainder of gp41 and all of gp120 from a wild type, X4-tropic env. Corresponding pseudoviruses (NL4-3-E1-CT, NL4-3-E51-CT, NL4-3-PIE1-CT and NL4-3-PIE2-CT) were made by co-transfecting HEK293T cells with NL4-3ΔEnv and constructs expressing the above chimeric Envs. Figures 14G, 14H, and 14I show that viruses pseudotyped with the NL4-3-PIE2-CT envelope are resistant to PIs even when the gag, and pol genes and most of the env gene, with the exception of gp41 CT, are wild type. Pseudoviruses made with the other chimeric envelopes demonstrated dose-response curves that were not significantly different than the wild type. Therefore, in some cases mutations in the gp41 CT are sufficient to confer PI resistance. Viruses harboring only these mutations would be erroneously identified as drug-susceptible clinical assays due to the exclusion of HIV-1 env region and the use of MLV-E in generating pseudoviruses. Taken together, these results demonstrate that an accurate assessment of PI resistance may require analysis of the env gene.
Figure 14: The effect of mutations in the env gene on PI resistance.

Mutations in HIV-1 Env alone can confer PI resistance. Eight HIV-1 variants were isolated from a patient with high-level resistance to PIs and passaged in vitro in the presence of increasing concentrations of DRV (51). Full-length env was cloned from passage 1 and passage 51 (E1 and E51). Pseudoviruses with WT, E1 or E51 Env and wild type HEK293T cells were co-transfected with an NL4-3ΔEnv vector expressing wild type gag and pol, and vectors expressing either the E1, E51 Env or a wild-type X4-tropic Env. Transfected cells were plated in 96-well plates and the indicated PIs (ATV, DRV, or LPV) were added. Two days after transfection, supernatants were used to infect CD4+ Lymphoblasts. Three days later, the infection was quantified as the percentage of cells expressing GFP+. (D,E,F) Envs cloned from patients who failed PI-containing regimens without evidence of major PI mutations confer PI resistance. Full-length env genes were cloned from two patients with viral loads >50 copies who were treated with PI-containing regimens and did not have evidence of PI-resistance mutations in the protease gene (PIE1 and PIE2). HEK293T cells were co-transfected with an NL4-3ΔEnv vector expressing wild type gag and pol and vectors expressing either the PIE1, PIE2, or a wild-type X4-tropic HIV-1 Env. Transfected cells were plated in 96-well plates and PIs were added. Two days after transfection, supernatants were used to infect CD4+ lymphoblasts. Three days later the infection was quantified as the percentage of cells expressing GFP. (G,H,I) Effect of mutations in the gp41 cytoplasmic tail on PI sensitivity. A chimeric, full length env was engineered with gp120 and most of gp41 with the exception of the CT NL4-3 and the CT of gp41 from PIE2 (NL4-3-PIE2-CT). HEK293T cells were co-transfected with an NL4-3ΔEnv vector expressing wild type gag and pol and vectors expressing either the NL4-3-PIE2-CT, or a wild-type X4-tropic envelope. Transfected cells were plated in 96-well plates, and PIs were added. Two days after the transfection, supernatants were used to infect CD4+ Lymphoblasts. Three days later, the infection was quantified as the percentage of cells expressing GFP. The panels on the first, second and third columns are dose-response curves for ATV, DRV, and LPV, respectively. Drug concentrations are normalized to the IC50 values for infectivity measured in (2) and are 13.6 nM, 23.6 nM, and 35.8 nM for ATV, DRV and LPV, respectively.
Figure 15: Effect of mutations in the Envs cloned from patients who failed PI-containing regimens without evidence of major PI mutations in the protease gene on PI resistance.

Full-length \textit{env} was cloned from two patients with plasma HIV-1 RNA levels >50 copies/ml who were on PI-containing regimens and did not have evidence of PI-resistance mutations in the protease gene (PIE1 and PIE2) as well as from a patient who is taking HAART with a plasma HIV-1 RNA level <50 copies (HAART) and a treatment-naïve chronic progressor (CP). 293T cells were co-transfected with an NL4-3ΔEnv vector expressing wild type \textit{gag} and \textit{pol} and with vectors expressing either of the above Env constructs or a wild-type X4-tropic HIV-1 Env. Transfected cells were plated in 96-well plates, and PIs were added. Two days after transfection, supernatants were used to infect CD4\textsuperscript+ lymphoblasts. Three days later, the infection was quantified as the percentage of cells expressing GFP.
Table 1: Patient characteristics

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<th>CD4 count (cells/µl)</th>
<th>Current Regimen</th>
<th>Known resistance mutations</th>
<th>Previous antiretroviral treatment history</th>
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</tbody>
</table>

\(^a\) AA: African American; C: Caucasian

\(^b\) Drug abbreviations: 3TC, lamivudine; ABC, abacavir; ATV/r, atazanavir boosted with ritonavir; AZT, zidovudine; d4T, stavudine; ddi, didanosine; DRV/r, darunavir boosted with ritonavir; EFV, efavirenz; FTC, emtricitabine; IDV, indinavir; LPV/r, lopinavir boosted with ritonavir; NFV, nelfinavir; RAL, raltegravir; TDF, tenofovir disoproxil fumarate.

\(^c\) Resistance mutations detected on genotypic analysis. Mutations are classified as resistance mutations according to Reference 19 and the Stanford University HIV Drug Resistance Database (http://hivdb.stanford.edu/). For protease, the listed mutations are considered minor resistance mutations for ATV. RT, reverse transcriptase; Pro, protease; Int, integrase.

\(^i\) Plasma HIV-1 RNA level and CD4 count determined 9 weeks prior to sampling for env cloning.

\(^k\) Plasma HIV-1 RNA level and CD4 count determined 2 weeks prior to sampling for env cloning.

\(^j\) Plasma HIV-1 RNA level and CD4 count determined same day as sampling for env cloning.
Table 2: Summary of the dose-response curve parameters for patient-derived Env clones.

<table>
<thead>
<tr>
<th>Pt.</th>
<th>Clone</th>
<th>X4/ R5&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Drug</th>
<th>$IC_{50}$ (nM)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Slope&lt;sup&gt;b&lt;/sup&gt;</th>
<th>$IP_{Cmax}$&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Fractional Change in $IP_{Cmax}$&lt;sup&gt;d,j&lt;/sup&gt;</th>
<th>Replication Capacity&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Selective Advantage&lt;sup&gt;f&lt;/sup&gt;</th>
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<tbody>
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<td>HXB2</td>
<td>X4</td>
<td>ATV</td>
<td>9.09±0.61</td>
<td>2.68±0.38</td>
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<td>7.21±0.42</td>
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<tr>
<td>CP</td>
<td>98rd12</td>
<td>R5</td>
<td>ATV</td>
<td>NA&lt;sup&gt;g&lt;/sup&gt;</td>
<td>NA&lt;sup&gt;g&lt;/sup&gt;</td>
<td>NA&lt;sup&gt;g&lt;/sup&gt;</td>
<td>NA&lt;sup&gt;g&lt;/sup&gt;</td>
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<td>2522</td>
<td>R5</td>
<td>ATV</td>
<td>NA&lt;sup&gt;g&lt;/sup&gt;</td>
<td>NA&lt;sup&gt;g&lt;/sup&gt;</td>
<td>NA&lt;sup&gt;g&lt;/sup&gt;</td>
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<td>NA&lt;sup&gt;g&lt;/sup&gt;</td>
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<td>ATV</td>
<td>NA&lt;sup&gt;g&lt;/sup&gt;</td>
<td>NA&lt;sup&gt;g&lt;/sup&gt;</td>
<td>NA&lt;sup&gt;g&lt;/sup&gt;</td>
<td>NA&lt;sup&gt;g&lt;/sup&gt;</td>
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<td>X4</td>
<td>ATV</td>
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<td>ATV</td>
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<td>ATV</td>
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<td>R5</td>
<td>DRV</td>
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<td>9.21±0.55</td>
<td>0.04±0.00</td>
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<td>R5</td>
<td>DRV</td>
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<td>R5</td>
<td>DRV</td>
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<tr>
<td>Pt.</td>
<td>Clone</td>
<td>X4/ R5</td>
<td>Drug</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; (nM)</td>
<td>Slope</td>
<td>HP&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Fractional Change in HP&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Replication Capacity</td>
<td>Selective Advantage</td>
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<td>DRV</td>
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<td>DRV</td>
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<td>0.17±0.01</td>
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<td>DRV</td>
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<td>DRV</td>
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<td>DRV</td>
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<td>IIPCmax</td>
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<td>Replication Capacity</td>
<td>Selective Advantage</td>
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1Coreceptor usage predicted by the Geno2Pheno HIV coreceptor usage prediction algorithm (68).
2Slope and IC50 were calculated by fitting a linear regression model to the plot of log((1- f_u )/f_u ) vs. log(D/IC50) (Equation 2). See Appendix 2 for details. Higher slope values are obtained in analysis that considers the upward inflection in the median effect plots (see reference 4).
3IIPCmax is the number of logs of inhibition of single round of infection at Cmax and was calculated using Equation 7 (Appendix 2).
4Fractional change in IIPCmax (ΔIIPCmax) for a given Env clone is calculated using Equation 9 (Appendix 2). A positive value indicates a drug-resistant clone, whereas a negative value indicates a more susceptible clone compared to the wild type.
5Replication capacity is the relative ability of a mutant virus to complete a single round of infection compared to the wild type in the absence of a drug. It is calculated according to Equation 11 (Appendix 2).
6Selective advantage (SA) is calculated using Equation 12 (Appendix 2).
7Clones were not analyzed for this drug.
8IIPCmax for the wild type HXB2 was calculated by combining the two dose-response curves for entry and post-entry steps, as described in Equations 3 and 4.
9The 95% confidence interval for Fractional change in IIPCmax (ΔIIPCmax) is calculated as [μ-1.96×σ, μ+1.96×σ], where μ is the mean and σ is the standard error of the mean for ΔIIPCmax. Clones with 95% confidence interval for ΔIIPCmax that does not include zero for all three drugs tested are in bold.
CHAPTER 4: CONCLUSION
Modern HAART regimens typically consist of three drugs. Each drug inhibits a subset of infection events on its own; however, in combination therapy, multiple log inhibition is achieved. Very high levels of inhibition can be achieved with combinations that include drugs targeting different steps in the life cycle (4). In this situation, drugs acting at downstream steps in the life cycle block viruses that were not inhibited by drugs acting at the previous steps. A full replication cycle requires a virus to clear multiple blocks from drugs acting at different steps. The low probability with which this occurs contributes to the success of HAART (4).

Among anti-HIV-1 drugs, PIs stand out. We and others have shown that in vitro, PIs exhibit superior antiviral activity (4,49-51). In addition, their dose-response curves are steep. Median effect plots of the dose-response relationship are non-linear and inflect upward as the concentration of the drug increases, resulting in extraordinarily high levels of inhibition at concentrations only slightly above the IC50. These unique pharmacodynamic features make PIs candidate drugs for monotherapy. In fact, PIs are the only class of antiretroviral drugs for which clinical trials have demonstrated that monotherapy is non-inferior to triple-drug therapy at maintaining suppression of HIV-1 replication (5,52).

In this study, we show that in effect, PIs act like multiple drugs in one. Using a novel, highly sensitive budding assay, we proved that immature particles can be released efficiently from cells treated with PIs. However, these immature virions produced in the presence of PIs are incapable of efficiently completing entry, reverse transcription, and post-reverse transcription steps.
A sensitive FRET-based virus-cell entry assay (37) allowed us to measure the inhibition of entry by PIs. Using pseudoviruses with a truncated gp41 CT allowed us to bypass the entry block and thereby isolate and measure the dose-response curves for PIs at the reverse transcription step. In addition, measuring GFP expression following infection with VSV-G-pseudoviruses, which are not inhibited at entry, allowed us to measure post-entry inhibition. Subtracting out the reverse-transcription inhibition from post-entry inhibition allowed us to mathematically obtain the post-reverse transcription inhibition by PIs. Finally, by combining the dose-response curves of PIs at the entry and post-entry steps, we were able to reconstruct the overall dose-response curves for the inhibition of infectivity by PIs. In doing so, we used the Bliss independence model (27), which is often used to predict the combined effect achieved by two drugs that act independently. The reconstructed dose-response curves matched those obtained experimentally. Importantly, in this analysis, no additional parameters were introduced to fit the dose-response curves. The predictions were based solely on experimental data for inhibition at individual steps, with the combined effect calculated using the Bliss independence model. We conclude that PIs achieve very high antiviral activity by blocking the viral life cycle at multiple steps. Similarly high levels of inhibition can be achieved by combinations of potent inhibitors of entry, reverse transcription and integration (4).

It is interesting that distinct IC_{50} values can be observed for inhibition of distinct life cycle steps even though the inhibition reflects the action of the same drug on the same target. PIs may block entry because cleavage of the Gag precursor protein renders the virion fusion-competent. PIs block post-entry steps because cleavages of the Gag-Pol precursor protein release the reverse transcriptase and integrase enzymes. Thus although
protease is the drug target in both cases, the substrate of the inhibited enzyme is different (Gag vs Gag-Pol). Cleavage of the Gag-Pol precursor protein generates three different enzymes, each of which is a multimer and each of which may be required in a different amount for successful completion of the life cycle (7). It is therefore not surprising that different levels of cleavage of these substrates may be required for each of the relevant steps. Hence the dose-response curves can be different.

Our results are consistent with previous studies that have separately noted effects of PIs on individual steps in the life cycle. The virions budding from PI-treated cells are morphologically aberrant and show reduced infectivity (50,51,53-55). This can be partially explained by lack of processing of Pr160Gag-Pol. This polyprotein is the precursor to the viral enzymes protease, RT, and integrase. In immature viral particles, the uncleaved Pr160Gag-Pol precursor exhibits significantly less reverse transcription activity compared to the fully cleaved form (14). Similarly, when virus-producing cells were treated with a first-generation protease inhibitor (A-77003), the resulting immature viruses produced 20 fold less early RT products in the infected cells compared to viruses produced by untreated cells (56). In addition, several groups have shown that immature virus particles with inactive protease (17) or Gag cleavage sites mutations (16,18) are inefficient in completing entry.

The clinical management of patients taking PIs is complicated by the fact that in clinical trials, the majority of virologic failures occur without evidence of genotypic or phenotypic resistance to these drugs (20-23). There are several potential explanations for this observation. One explanation is simply that the patients are non-adherent. If the non-adherence applies to all drugs in the regimen, then the patients are expected to fail
with wild type virus. This virus will show drug susceptibility in all types of resistance tests. Another explanation is that as concentrations of PIs decrease with non-adherence, their inhibitory potential rapidly falls below the level required for the selection of resistance mutations. In this situation, mutations conferring resistance to other drugs in the regimen may arise (23), but the virus will demonstrate susceptibility to PIs in all forms of resistance testing. This hypothesis was recently shown to be plausible using a mathematical model that combines viral dynamics, evolutionary principles, and pharmacokinetic and pharmacodynamics properties of the drugs (57). The lack of PI resistance in this scenario is supported by the clinical observation that patients can be successfully treated with the same PI if adherence is restored. For example, Kempf et al. observed that with improved adherence, 25 out of 27 patients who previously failed LPV/r containing regimens achieved virologic suppression when put back on the same PI-containing therapy (58).

An additional mechanism for PI failure without apparent resistance is described in this study. It involves mutations in the \textit{env} gene that significantly impact the antiviral activity of PIs. This mechanism is important because it leads to actual resistance to the PIs. In this situation, viral isolates will show PI resistance in \textit{in vitro} assays, but this resistance may not be apparent in standard commercial resistance assays. Patient-specific changes in the \textit{env} gene are not considered in standard clinical assays for genotypic or phenotypic resistance. In this study, we have shown that a significant fraction of the inhibition exerted by PIs is due to effects on HIV-1 entry. For DRV 46% of the 9.2 logs of inhibition at $C_{\text{max}}$ was due to the inhibition of entry, compared to only 22% at the reverse transcription step. We hypothesized that escape from the entry
inhibition of PIs could be a pathway by which viruses acquire PI resistance. To test this hypothesis, we first showed that known drug resistance mutations in the protease gene achieve their overall resistance phenotype by altering both the entry and post-entry dose-response curves. However, one of the standard phenotypic assays does not detect this change in the inhibition of entry because it utilizes pseudoviruses with MLV-Env instead of HIV-1 Env. MLV-Env requires cleavage of its short cytoplasmic tail by HIV-1 protease to mediate fusion (Figure 13 and references (46). Thus, the dose-response curves of PIs obtained using the MLV-Env-pseudotyped particles do not include the HIV-1 inhibition of PIs at the entry step and are confounded by the effect of PIs on a clinically irrelevant substrate, the MLV-Env. Given the additional complexity introduced by the use of MLV-Env, it is possible that the phenotypic assay may either overestimate or underestimate the actual degree of resistance. It is also important to point out that PI resistance without mutations in protease could be due in part to mutations in Gag that directly or indirectly affect the protease cleavage sites (59-65).

We also show here for the first time that mutations in the env gene of HIV-1 can confer PI-resistance even when the gag and pol genes are wild type. We cloned full-length env genes from the circulating virus of patients who failed PI-containing regimens with no known major PI-associated resistance mutations as determined by standard genotypic analysis. Pseudoviruses generated with these Envs and a wild type backbone, including wild type Gag and Pol, demonstrated significant PI resistance. This was especially apparent at higher drug concentrations where the effect on HIV-1 entry becomes important. We localized the relevant drug-resistance mutations in one of these patients to the gp41 CT using a chimeric env gene with gp120 and most of gp41, except
the CT, from the wild type NL4-3 strain and the CT from the patient isolate. This chimeric Env fully recapitulated the PI-resistance phenotype. A molecular basis for this phenomenon has been provided by a recent fluorescence nanoscopy study showing that the clustering of Env spikes that is necessary for entry may require protease-mediated cleavage of the Gag precursor protein (66). Some mutations in the gp41 CT may preclude interactions with the uncleaved Gag precursor that normally inhibit clustering and thus entry. In the case of three other PI-resistant Env clones, transfer of the CT to the NL4-3 background did not transfer resistance. In these instances, resistance may involve the complex dynamics of the entire Env trimer. A further caveat is that interactions between uncleaved Gag precursor and the gp41 CT might differ between a patient-derived Gag precursor and NL4-3-derived Gag precursor. Future biochemical studies of the interactions between the gp41 CT and Gag may provide a molecular explanation for the forms of PI resistance described here. Because of the unstructured nature and high sequence variability of the env CT, the detection of this form of resistance may require functional tests rather than simple genotypic analysis.

By experimentally isolating each relevant step of the HIV-1 life cycle and measuring the dose-response curves for PIs at each step, we have provided here a mechanistic explanation for the unique pharmacodynamics and exceptional efficacy of PIs. In addition, our analysis pointed to the previously ignored env gene as a possible site of PI-resistance mutations. We showed that env genes cloned from primary viral isolates from patients who failed PI-containing regimens with virus classified as wild type virus could confer PI resistance. This suggests that in some cases clinicians may be falsely
reassured by relying on current commercial genotypic and phenotypic resistance assays, which do not take into account mutations in the \textit{env} gene. Additional studies will be needed to determine how common this phenomenon is. If these \textit{env} mutations turn out to be common, modifications in current clinical assays may be necessary to accurately quantify PI resistance.

Our results may also provide a blueprint for development of new therapeutics for other pathogens. For drugs such as PIs that target proteins influencing multiple functions that a pathogen needs in order to establish a productive infection, the inhibitory effects will multiply to achieve much greater overall inhibition compared to classes of drugs that inhibit a single step in the pathogen’s life cycle.
APPENDIX 1

Here we show that independent inhibition by a single drug of two distinct steps (A and B) in the viral life cycle results in a non-linear median effect dose-response curve that infects upward. At high drug concentrations, the slope ($m$) of the overall dose-response curve approaches the sum of the slopes of the dose-response curves at steps A and B.

Suppose PIs inhibit two independent steps of life cycle, A and B. Let $I_A$ be the $IC_{50}$ for inhibition of step A and $I_B$ by the $IC_{50}$ for inhibition of step B.

From equation 1, we have for step A:

$$f_{uA} = \frac{1}{1 + \left(\frac{D}{I_A}\right)^{m_A}} = \frac{I_A^{m_A}}{I_A^{m_A} + D^{m_A}}$$

For step B:

$$f_{uB} = \frac{1}{1 + \left(\frac{D}{I_B}\right)^{m_B}} = \frac{I_B^{m_B}}{I_B^{m_B} + D^{m_B}}$$

By Bliss independence,

$$f_u = f_{uA} f_{uB}$$
\[
\frac{f_a}{f_u} = 1 - \frac{f_u}{f_u} = 1 - f_u A f_u B = \frac{1}{1 - (I_A^m + D^m_A)(I_B^m + D^m_B) - 1} \]

\[
\log \left( \frac{f_a}{f_u} \right) = \log \left[ \frac{(I_A^m + D^m_A)(I_B^m + D^m_B)}{I_A^m I_B^m} - 1 \right] \]

\[
\log \left( \frac{f_a}{f_u} \right) = \log \left[ \frac{I_A^m I_B^m + D^m_A I_B^m + I_A^m D^m_B + D^m_A D^m_B}{I_A^m I_B^m} - 1 \right] \]

\[
\log \left( \frac{f_a}{f_u} \right) = \log \left( \frac{D^m_A I_B^m + I_A^m D^m_B + D^m_A D^m_B}{I_A^m I_B^m} \right) - \log \left( I_A^m I_B^m \right) \]

At \( D >> I_A, I_B \):

\[
\log \left( \frac{f_a}{f_u} \right) \approx \log \left( D^m_A D^m_B \right) - \log \left( I_A^m I_B^m \right) \]

\[
\log \left( \frac{f_a}{f_u} \right) \approx (m_A + m_B) \log(D) - \log \left( I_A^m I_B^m \right) \]

Thus at high \( D \), the median effect plot of \( \log (f_a/f_u) \) vs \( \log D \) for the inhibition on infectivity will approach a slope of \( m_A + m_B \).
APPENDIX 2

Formulae used in Table 1 and the calculation of error.

\( F_u \) and \( D \) are as defined previously (Equation 2) and \( IC_{50} \) is the concentration of drug that achieved 50% inhibition of one round of replication in reference (2). To calculate the \( IC_{50} \), the following formula was used:

\[
\log \left( \frac{\left( 1 - f_u \right)}{f_u} \right) = m \log \left( \frac{D}{IC_{50}} \right)
\]

Equation 2

\[
\log \left( \frac{\left( 1 - f_u \right)}{f_u} \right) = m \log \left( \frac{D}{IC_{50}} \times \frac{IC_{50}'}{IC_{50}''} \right)
\]

\[
= m \log \left( \frac{D}{IC_{50}} \right) + m \log \left( \frac{IC_{50}'}{IC_{50}''} \right)
\]

Thus, plotting \( \log \left( \frac{\left( 1 - f_u \right)}{f_u} \right) \) vs \( \log \left( D/IC_{50} \right) \) will approximately linearize the dose-response curve. The y-intercept (\( b \)) is equivalent to \( m \log \left( \frac{IC_{50}'}{IC_{50}''} \right) \). Therefore,

\[
b = m \log \left( \frac{IC_{50}'}{IC_{50}''} \right)
\]

Equation 5

\[
IC_{50} = \frac{IC_{50}'}{10^b/m}
\]

Microsoft Excel© LINEST function was used to calculate the line of best fit where unknown Y’s and unknown X’s were selected to be \( \log \left( \frac{\left( 1 - f_u \right)}{f_u} \right) \) and \( \log \left( D/IC_{50} \right) \), respectively. In addition to the slope (\( m \)) and the y-intercept (\( b \)), this function returns the error in these values \( \delta m \) and \( \delta b \).

The following formulae were used to calculate the other parameters in Table 1. The symbol \( \delta \) indicates the error in each parameter.
\[ \delta IC_{50} = \frac{IC_{50} \left( \frac{b}{m} \right) \left( \frac{\delta b}{b} \right)^2 + \left( \frac{\delta m}{m} \right)^2}{0.434} \]  
Equation 6

\[ II_{IP_{c_{\text{max}}}} = \log \left( 1 + \left( \frac{C_{\text{max}}}{IC_{50}} \right)^m \right) \]  
Equation 7

\[ \delta II_{IP_{c_{\text{max}}}} = \delta m \log \left( \frac{C_{\text{max}}}{IC_{50}} \right) \quad (C_{\text{max}} >> IC_{50}) \]  
Equation 8

The fractional decrease in the inhibitory potential of the drug at \( C_{\text{max}} \) (\( \Delta II_{IP_{c_{\text{max}}}} \)) for each mutant clone compared to the wild type can be calculated as:

\[ \Delta II_{IP_{c_{\text{max}}}} = 1 - \frac{II_{IP_{\text{clone,c_{\text{max}}}}}}{II_{IP_{\text{wt,c_{\text{max}}}}}} \]  
Equation 9

where the inhibitory potential of the drug against the mutant clone at \( C_{\text{max}} \) is denoted as \( II_{IP_{\text{clone,c_{\text{max}}}}} \). The corresponding value for the wild type envelope is denoted as \( II_{IP_{\text{wt,c_{\text{max}}}}} \).

The error in \( \Delta II_{IP_{c_{\text{max}}}} \), \( \delta(\Delta II_{IP_{c_{\text{max}}}}) \) is calculated using Equation 10.

\[ \delta(\Delta II_{IP_{c_{\text{max}}}}) = \Delta II_{IP_{c_{\text{max}}}} \left( \frac{\delta II_{IP_{\text{clone,c_{\text{max}}}}}}{II_{IP_{\text{clone,c_{\text{max}}}}}} \right)^2 + \left( \frac{\delta II_{IP_{\text{wt,c_{\text{max}}}}}}{II_{IP_{\text{wt,c_{\text{max}}}}}} \right)^2 \]  
Equation 10

The Replication Capacity of a clone (\( RC_{\text{clone}} \)) is calculated as follows:

\[ RC_{\text{clone}} = \frac{f_{\text{clone}}}{f_{\text{wt}}} \]  
Equation 11

where \( f_{\text{clone}} \) and \( f_{\text{wt}} \) are defined as the fitness of the mutant clone and the wild type, respectively. Experimentally, they are measured as the percentage of GFP\(^+\) cells observed when CD4\(^+\) T cells are infected with each virus in the absence of any drug.

The Selective advantage of a given clone at a given dose (\( D \)) of the drug, is defined as follows:
\[ SA = RC_{clone} \times 10^{(\text{IC}_{50} - \text{IC}_{50})} \]  

Equation 12

**APPENDIX 3**

*Reagents and viruses*

PIs were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. To facilitate a comparison of PIs with different potencies, drug concentrations were normalized by \( IC_{50} \) values determined in a previous study (2). The \( IC_{50} \) values are given in the legend to Figure 4. Drug concentrations (\( D \)) were plotted as \( \log(D/IC_{50}) \). In various experiments, drugs were used at concentrations ranging from 0.01 to 100 x \( IC_{50} \) (0.136 nM – 1.36 µM for ATV, 0.236 nM – 2.36 µM for DRV, and 0.358 nM – 3.58 µM for LPV). CCF2-AM, and Benzonase are commercially available from Invitrogen and Sigma, respectively. Beta-lactamase antibody was purchased from Abcam and anti-p24 antibody from Santa Cruz Biotechnology.

Viruses selected *in vitro* for DRV resistance (E1 and E51) were a gift of Dr. Hiroaki Mitsuya at the National Institutes of Health (NIH).

*Plasmids and Vector Constructs*

The plasmid pMM310 expresses *Escherichia coli* beta-lactamase fused to the viral accessory protein Vpr. It was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: pMM310 (Cat#11444) from Dr. Michael Miller. Beta-lactamase expression vector was obtained by inserting a stop codon at the end of *beta-lactamase* open reading frame (ORF) via site-directed mutagenesis (Stratagene).
pNL4-3ΔEnv expresses HIV-1 with GFP in the open reading frame (ORF) of the env gene (2). The RT-deficient construct (D185N) and the protease-deficient construct were obtained from pNL4-3 ΔEnv via site-directed mutagenesis. The LPV resistance mutations V82A and V82F were introduced into the protease gene via site-directed mutagenesis.

The plasmid pSV-A-MLV-env expresses the amphotropic Murine Leukemia Virus env gene (MLV-E) and was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: SV-A-MLV-env from Dr. Nathaniel Landau and Dr. Dan Littman.

Analysis of viral budding

HEK 293 T cells were co-transfected with NL4-3 Δ Env GFP and a vector expressing an X4-tropic Env using the Lipofectamin 2000 reagent (Invitrogen) according to the manufacturer’s protocol. 6 hours after the transfection the cells were separated by trypsinization and distributed in 96-well plates, and PIs were added. Drugs were diluted in 50% human serum (HS) and the cell medium also contained 50% HS to recapitulate the effect of protein binding in vivo. At 48 hours after transfection, cell-free supernatant was treated with 1 unit of Benzonase (Sigma) for 15 minutes in 37° to degrade free, extra-virion RNA. Viral RNA was extracted using QIAamp Viral RNA Mini Kit (Qiagen). Oligo-dTs were used as primers for reverse transcription using the Superscrip III enzyme (Invitrogen). Real-time PCR was performed using TaqMan® PreAmp Master Mix using the following primer-probe pairs:

Up-stream: CAGATGCTGCATATAAGCAGCTG

Down-stream: TTTTTTTTTTTTTTTTTTTTTTTTTTTGAAGC
Probe: CCTGTACTGGGTCTCTCTGG

Viral fusion assay

A modified version of the previously described assay for viral entry based on fluorescence resonance energy transfer (FRET) (37) was utilized. Briefly, HEK 293T cells were co-transfected with NL4-3 ΔEnv, BLAM-Vpr and an envelope-expressing construct depending on the experiment. 6 hours after transfection, the cells were detached by trypsinization and were distributed into 96-well plates, and PIs were added. Drugs were diluted in 50% HS and cell media contained 50% HS, as described above. After 48 hours, virus-containing supernatants were used to infect primary CD4+ T lymphoblasts via spinoculation (2 hrs, 1200 x g, 25°C). After allowing the entry to occur in 37°C for 2 hours, CD4+ T cells were incubated in CCF2-AM-containing media for one hour at room temperature. Cells were then washed in RPMI and incubated overnight in CO2-independent media (Invitrogen) at room temperature. Green to blue color change was detected by flow cytometry to quantify viral entry.

Infectivity assay

We utilized a GFP single-round infectivity assay described before. Briefly, HEK 293T cells were co-transfected with NL4-3ΔEnv, BLAM-Vpr and an envelope-expressing construct depending on the experiment. An X4 Env (from NL4-3) was used for most experiments because X4-pseudotyped viruses give higher levels of infection of CD4+ T lymphoblasts in the infectivity assay and therefore provide a higher dynamic range for assessing drug inhibition. However, we have also used an R5 Env (from SF162). Dose response curves for PI-mediated inhibition of infection by pseudoviruses carrying the reference X4 and R5 Envs were superimposable (Figure 16). In some experiments,
patient-derived Envs were used. Six hours after transfection, 293T cells were detached by trypsinization and were distributed into 96-well plates, and PIs were added. Drugs were diluted in 50% HS and cell media contained 50% HS, as described above. After 48 hours, virus-containing supernatants were used to infect primary CD4⁺ T lymphoblasts via spinoculation (2 hrs, 1200 x g, 25°C). CD4⁺ T lymphoblasts were prepared by stimulation of peripheral blood mononuclear cells from healthy blood donors with phytohemagglutinin and interleukin-2 for 3 d followed by magnetic bead purification as previously described (2). The PI effect is exclusively at the virus production step and the presence of PIs during the actual infection of CD4⁺ T cells has no effect on infectivity (Figure 17). Cells were incubated at 37°C for 3 days to allow completion of viral life cycle and expression of GFP, which was then analyzed using flow cytometry.

Envelope cloning and generation of chimeric envelopes

Full-length HIV-1 env genes from patient isolates or samples were cloned into an expression vector as described previously (67). Based on the Geno2Pheno HIV coreceptor usage prediction algorithm (68), the E1 and E51 isolates and isolates from patient PIE5 have predicted X4 tropism while the rest of the env clones have predicted R5 tropism (Table 1). Overlap extension polymerase chain reaction was utilized to make constructs expressing the chimeric Env proteins with the CT of gp41 from the PI-resistant viruses E-1, E-51, PIE1, or PIE2, and the remainder of gp41 and all of gp120 from a wild type, X4-tropic envelope (NL4-3). No additional nucleotides were inserted in the junction site during the cloning.
Study Subjects

Primary CD4+ T cells were isolated from peripheral blood of healthy or HIV-1 infected adults. This study was approved by the Johns Hopkins Institutional Review Board and all study participants provided informed consent.
Figure 16: The effect of Env tropism on PI dose-response curves.

HEK 293T cells were co-transfected with an NL4-3ΔEnv vector expressing wild type gag and pol and with vectors expressing either R5- or X4-tropic HIV-1 Envs. Transfected cells were plated in 96-well plates, and the indicated PIs were added. Two days after transfection, supernatants were used to infect CD4+ lymphoblasts. Three days later, the infection was quantified as the percentage of cells expressing GFP.
HEK 293T cells were co-transfected with an NL4-3ΔEnv vector expressing wild type gag and pol and with vectors expressing X4-tropic HIV-1 env. Two days later, supernatants were used to infect CD4+ lymphoblasts. ATV was present at the indicated concentrations either during virus production by HEK 293T cells, during the infection of CD4+ T lymphoblasts, or both during virus production and infection of CD4+ T lymphoblasts.


Curriculum Vita

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Other Professional Experience:
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Scholarships:
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Academic Honors:
2012 Young Investigator Award -Conference on Retroviruses and Opportunistic Infections (CROI)
2011 Grand Challenges Explorations Grant -Gates Foundation
2011 Kelly Award –Johns Hopkins Institute for Basic Biomedical Sciences
2006 Engineering Science Award of Excellence, University of Toronto
2005 Natural Science and Engineering Research Council (NSERC) Undergraduate Award.
2004 Etkin Medal of Excellence in Fluid Dynamics and Solid State Mechanics –University of Toronto
2004 J. Frank Guenther Scholarship -University of Toronto
2004 NSERC Undergraduate Award -University of Toronto
2003 Outstanding Achievement Award in Recognition of Excellence in Biology, Department of Botany and Zoology, University of Toronto

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**Book Chapters:**


**Presentations:**


3) **Rabi SA**. What do Protease Inhibitors Do? 14th annual HIV Drug Resistance Program Think Tank Meeting. National Cancer Institute, Frederick, MD. 2011. Invited speaker


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Summer 2011/2012 Medical school scholarly concentration course. Teaching Assistant
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