DIFFERENTIAL IMPACT OF HIV-1 PROTEASE INHIBITORS ON SUBSETS OF CD4+ T-LYMPHOCYTES

By

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ABC	ATP-Binding Cassette
ART	Antiretroviral Therapy
BCRP	Breast Cancer Resistance Protein
BLQ	Below Limit of Quantitation
HIV-1	Human Immunodeficiency Virus Type 1
IN	Integrase
MRP	Multidrug Resistance Transporter Protein
NNRTI	Non Nucleoside Reverse Transcriptase Inhibitor
NRTI	Nucleoside Reverse Transcriptase Inhibitor
p-gp	p-glycoprotein
PI	Protease Inhibitor
PI^{res}	Protease Inhibitor Resistant
PI^{sen}	Protease Inhibitor Sensitive
PR	Protease
Rh-123	Rhodamine 123
RT	Reverse Transcriptase
R5	CCR5
X4	CXCR4

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Human immunodeficiency virus type 1 (HIV-1), the causative agent of acquired immunodeficiency syndrome (AIDS), affects nearly 40 million people. HIV-1 targets cells expressing CD4 and one of two major chemokine coreceptors, CXCR4 (X4) or CCR5 (R5). HIV-1 enters into cells via sequential interaction with CD4 receptor and either X4 or R5 coreceptors. Protease Inhibitors (PI) are a class of antiretroviral agents used to treat HIV-1 infected individuals. PIs are substrates for six known efflux transporters, most notably p-glycoprotein (p-gp). P-gp is a member of the ATP Binding Cassette (ABC) efflux transporter family, which displays highly variable expression among lymphocytes.

Lymphocytes are a primary target of infection and represent a heterogeneous population of cells that differ in receptor and coreceptor expression, activation state, surface expression of activation markers, and effector or memory function. Previous studies in our laboratory determined that significantly more PI is required to inhibit viral replication for otherwise identical viruses targeted to R5 versus X4 expressing T lymphocytes.

The hypothesis is that differences in efflux kinetics and peak intracellular PI levels in subsets of HIV-1 target cells within the lymphocyte population are responsible for requirement of significantly higher IC_{50} in the R5 versus X4 expressing lymphocytes. Three questions were

posed relative to subsets of CD4+ T lymphocytes to test the hypothesis: Optimal conditions at which to observe peak intracellular PI levels and efflux kinetics, receptor expression profile, and Rh-123 assay to observe p-gp activity.

Results are as follows: Using the Rh-123 assay to observe p-gp activity, it was determined that a subset of lymphocytes that are R5+/CD4+/CD45RA-/CD45RO+ displayed p-gp activity comparable to the highest known p-gp activity in any human cell, NK cells. Cells expressing CD4 and R5 are targets for HIV-1 infection by an R5 using virus. The population of cells displaying high p-gp activity represents one of the populations for R5 infection within the lymphocyte population, demonstrating a link between p-gp activity in the R5+/CD4+/CD45RA/CD45RO+ cells and the requirement for more drug in viruses targeted to R5 expressing T lymphocytes.

Although PI are substrates for other efflux transporters, and p-gp is not the sole determining factor for efflux and bioavailability, the conclusion is that p-gp activity is higher in a subset of the R5 target cells relative to cells representing the X4 HIV-1 targets. As p-gp activity is linked directly to intracellular bioavailability of PI, we can also conclude that these data demonstrate a link between p-gp activity and differences in IC_{50} for HIV-1 targeted to R5 versus X4 expressing T lymphocytes. These data may have implications for *in vivo* replication dynamics, therapy outcome, persistence of R5 infection, and viral reservoirs for drug resistant viruses emerging under the selective pressure of suboptimal levels of drug.

Suboptimal levels of ART is an ongoing problem in treating HIV-1 infection and presents obstacles including emergence of resistant mutations and clearance of virus from infected cells with low bioavailability Understanding dynamics of virus/host/drug interaction and identifying

mechanisms for decreased bioavailability of ART can lead to design of novel therapeutics to increase intracellular concentrations of drug.

CHAPTER 1
INTRODUCTION AND BACKGROUND

Human Immunodeficiency Type 1

HIV-1 is a retrovirus belonging to the genus Lentivirus. Infections by lentiviruses are characterized by remarkably complex interactions with the host and a chronic course of disease. Common features of disease include long and variable incubation periods, persistent viral replication, neurologic manifestations, and destruction of specific hematologic or immunologic cells [29, 45,100].

All lentiviruses exhibit a common morphogenesis and morphology, a tropism for macrophages, extensive genetic and antigenic variability, and the presence of additional regulatory genes not found in other groups of retroviruses. Lentiviruses have been isolated from several animal species including sheep, goats, horses, cattle, cats, monkeys, and humans. HIV-1 and HIV-2 are the only known human lentiviruses [29].

Since its identification in the early 1980's, Human Immunodeficiency Virus (HIV), the causative agent of Acquired Immune Deficiency Syndrome (AIDS), has escalated to a global pandemic. An estimated 39.5 million people are living with HIV-1 and 2.9 million people died of AIDS-related illnesses in 2006. There were 4.3 million new infections in 2006 with 2.8 million (65%) of these occurring in sub-Saharan Africa and important increases in Eastern Europe and Central Asia, where there are some indications that infection rates have risen by more than 50% since 2004 (WHO, 2006). The disease is characterized by susceptibility to opportunistic infection and a severe and steady decline in $CD4^+$ T cells [29, 45,48,100].

Groups and subtypes according to geographical location classify HIV-1. Group M (major) contains subtypes A-K, and is most prevalent worldwide. The most common subtypes in group

M are subtype B, which predominates in North America, Europe, parts of South America and India, subtype C which predominates in sub-Saharan Africa, and subtype E which predominates in Southeastern Asia. Two new groups, N (new) and O (outliner), have been identified in Africa and Eastern Europe (Figure 1-1) [29,92].

HIV-1 Genomic Organization

HIV-1 is a sense strand RNA virus which packages 2 copies of a positive-sense RNA strand genome of approximately 10,000 nucleotides (10 kb) into each virion. The genome is flanked by two identical long terminal repeats (LTR) and contains the three major genes *gag, pol,* and *env* as well as accessory proteins Vif, Vpr, Vpu, Tat, Rev, and Nef. The structural proteins matrix ($p17^{MA}$), capsid ($p24^{CA}$), nucleocapsid ($p7^{NC}$), and p6, are encoded in *gag*. Enzymes protease (PR), reverse transcriptase (RT), and integrase (IN) are encoded in *pol*. The *env* gene encodes the envelope glycoproteins gp120 and gp41, which are required for viral attachment and entry (Figure 1-2, 1-3). [29, 45,92,100]

HIV-1 Life Cycle

HIV-1 gp120 on the surface of the virion binds to CD4 receptor on the host cell. This causes conformational changes in gp120, which then expose coreceptor-binding sites, and cellular chemokine coreceptors CCR5 or CXCR4 are engaged. After attachment, the viral envelope (trimeric complexes of glycoproteins gp120-gp41) fuse with the lipid membrane of the target cell, releasing the viral core into cytoplasm of the host cell. Viral RNA is reverse transcribed into double-stranded DNA via viral enzyme RT. The DNA translocates to the nucleus and integrates into the host genome via viral enzyme IN. Transcription of the RNA and translation of gene products occur next, followed by assembly at the plasma membrane, budding of the newly formed virion, and protease processing resulting in a mature, infectious virion (Figure 1-4). [27, 29, 45,92,100]

Targets of HIV-1 Infection

HIV-1 entry into cells is mediated by target cell expression of CD4 and appropriate chemokine coreceptor, primarily CXCR4 or CCR5. CD4/CCR5 cells include macrophages ($t_{1/2}$ weeks) and a small subset of activated memory T lymphocytes ($t_{1/2}$ upon primary exposure to antigen = years, $t_{1/2}$ upon secondary exposure to antigen = days) while CXCR4 is expressed by most CD4 T lymphocytes, transformed lymphocytic or monocytic lineages, and macrophages. R5 viruses are consistently macrophage tropic (M-tropic) and display M-R5 phenotype, while X4 viruses display heterogeneous phenotypes. The majority of primary X4 viruses replicate in both macrophages and T cell lines, use CXCR4 either alone or in combination with CCR5 and are dual (D)-tropic, whereas X4 viruses which do not exhibit tropism for macrophages, but maintain ability to replicate in T cell lines, are T-X4 phenotypic. Viruses that can productively replicate in R5 expressing lymphocytes, but not macrophages, are termed L-R5 (Table 1-1). [8, 14, 17,29,32,45, 48, 49, 80]

R5 viruses predominate early in infection, whereas in ~ 50 % of patients, X4 viruses emerge and are associated with rapid progression to AIDS. Lymphocytes represent the primary target of HIV-1 infection, are a sentinel part of the adaptive immune response, and function to circulate between the peripheral blood and secondary lymphoid organs until antigen is encountered. Macrophages are part of the innate immune response, are phagocytic, and represent a small fraction of infected cells (Table 1-1). [8,29, 48,100-101]

Antiretroviral Therapy (ART)

Four classes of ART are used to control HIV-1 infection: entry inhibitors, nucleoside reverse transcriptase inhibitors (NRTI), non-nucleoside reverse transcriptase inhibitors (NNRTI), and protease inhibitors (PI). Other classes of drugs currently in clinical trials to treat HIV-1 infection include 1. Immune based therapies focused on CD4 T-cell reconstitution via

administration of T-cell proliferative cytokines, notably IL-2, and 2. Integrase inhibitors, which function to inhibit HIV-1 integration into the host cell genome. [11,29,100,111,116]

Entry Inhibitors and Mechanism of Action

There are two FDA approved entry inhibitors: T-20, a fusion inhibitor, and Selzentry, a CCR5 inhibitor. Other possibilities currently being studied for entry inhibitors include soluble CD4, siRNA directed silencing of CD4 mRNA expression, inhibition of chemokine coreceptor engagement by HIV-1 (targeting other binding sites on CCR5 than Selzentry), and molecules that target the envelope-CD4 interaction (Figure 1-5). [11, 29, 100,116]

T-20

Fusion between the virus and the host cell is a critical and necessary step in the viral life cycle. Without this event, release of the viral core into the host cell cytoplasm cannot occur. T-20 was approved by the U.S. Food and Drug Administration (FDA) in 2003 and is a 36kDa protein which functions as a dominant negative molecule, inhibiting formation of the hairpin structure necessary for fusion to occur (Figure 1-6). Disadvantages of T-20 treatment for HIV-1 infection include cost of synthesis, the large amount of T-20 required for efficacy, and the size of the molecule, which prohibits oral route, and must be injected. Clinical trials and studies conducted after FDA approval have demonstrated that T-20 is most efficacious when administered in combination with multiple drugs for which the virus is sensitive, but displayed limited ability to reduce viral loads when administered alone or in combination with drugs that the virus was resistant to. This correlates the use of T20 with administration of drugs that the virus is sensitive to, potentially limiting the circumstances that T20 can be successfully used (Figure 1-5). [11, 29,111,116]

Selzentry

Selzentry is a CCR5 inhibitor that was approved by the FDA in August 2007. The molecule inhibits viral entry via binding to CCR5 coreceptor, inhibiting the receptor/coreceptor viral envelope interaction required for HIV-1 entry into the host cell. Selzentry provides an advantage over other drugs, presenting an option for treatment of HIV-1 infected individuals with multiple drug resistance to other ART, and is approved for these individuals. Little is known about the long-term effect of Selzentry treatment, and thus is not approved for HIV-positive patients who have drug sensitive strains at this time. [111,116]

Reverse Transcriptase Inhibitors and Mechanism of Action

The two classes of Reverse Transcriptase Inhibitors are NRTI and NNRTI. NRTI were the first anti-HIV drugs used clinically, and require intracellular phosphorylation for activation. NRTI compete with endogenous nucleotides for incorporation into the growing viral DNA strand, however they lack a 3´ hydroxyl terminus, thus incorporation of the NRTI into the growing DNA strand results in termination of the DNA strand, as the next phosphodiester bond is not formed (Figure 1-6). [29,100,115]

NNRTI do not require intracellular phosphorylation for activation, have a limited effect on other cellular enzymes, and bind directly to RT, inhibiting its enzymatic activity (Figure 1-6). [29,100,116]

Protease Inhibitors and Mechanism of Action

PI target HIV-1 PR, an aspartic protease comprised of two 99 amino acid monomers, which are only active in homodimeric form. PR cleaves Gag and Gag-pol, resulting in a mature infectious virion. PIs are competitive inhibitors, which compete for binding in the active site with the natural substrate. Once bound, PI cannot be cleaved, resulting in inactivation of the enzyme (Figure 1-7.) [25-27, 29,100,116]

There are currently ten PI approved by the FDA for treatment of HIV infection, with the most recent FDA approval occurring in June, 2006, for TMZ-114. All PI belong to the competitive small-molecule inhibitor family of drugs. [29,100,40,116] Two experimental PI which have not yet been evaluated by the FDA are in clinical trials, and other PI in various stages of development exist. [116] They include:

- Agents which irreversibly modify the active site aspartates, resulting in diminished requirement for high concentration of inhibitor and possibly reducing development of resistance mutations
- Agents targeting dimer interface of the enzyme, preventing formation of the homodimer required for activity
- Defective PR monomers which function as dominant negative macromolecular inhibitors, forming heterodimeric complexes with wt PR monomers

Resistance to Antiretroviral Therapy

Resistance is conferred by multiple mechanisms. [1,6,9-10,12,20,24,25-27,29,40,49-50,61,63,100,117]

- Viral RT error rate (mutation rate is approximately 1 in 10^4 errors per base incorporated; one new mutation is introduced into each new copy of the genome), allowing for rapid accumulation of mutations in virus-encoded drug targets (RT, PR, Env)
- Mutations which reduce sensitivity to PI are found in patients naïve to PI therapy
- Accumulation of protease inhibitor resistant (PI^{res}) mutations (due to suboptimal drug levels)
- Cross Resistance

Interaction Between Drug, Cells, and Virus

The goal in treating HIV-1 infection is to delay progression to AIDS by controlling viral replication and preventing or reversing immune deficiency. The end result of administration of a PI containing regimen *in vivo* is far more complicated than eradication of virus via antiviral therapy. Many parameters impact therapy outcome and disease progression, and involve a complex interplay between drug, cells, and virus.

Bioavailability of ART

Bioavailability of ART is dependent of multiple factors including: [1,2,4,5,6,9,12,18-22,35,37,40,42,44,51,56,57,60,63-66,68,70-73,76,77,81,93,94,98,100,104,106,107,109,112]

- Route of administration: oral (Figure 1-8) versus injection
- Percent plasma protein binding of drug (which impacts amount of free drug available to enter cells, and efflux kinetics of drug)
- Percent of drug bound to intracellular proteins (which impacts their ability to inhibit viral replication)
- Size of drug
- Lipophilicity of drug
- Affinity of drug for efflux transporters
- Expression of efflux transporters in HIV-1 infected cells
- Variable efflux transporter expression between patients (7-fold)
- Ability of drug to modulate efflux transporter expression
- Drug metabolism in the intestinal lumen (applicable for orally administered drugs)
- Co-administration of other ART, which may boost the bioavailability or compete for binding to efflux transporters
- Polymorphisms of the MDR1 gene
- Affinity of the drug for the binding pocket of the efflux transporter

Efflux transporters for which PI are substrates

There are six known efflux transporters for which PI are substrates, which display differential expression, and activity across cell types (Table 1-2). [1,2,6,12,19,22,33,37,51,56-60,65,71,75,77,81,93,106] P-glycoprotein (p-gp/MDR1), multidrug resistance transporter protein 1 (MRP1), MRP2, MRP5 and BCRP (Breast Cancer Resistance Protein) are expressed in lymphocytes. P-gp, MRP1, MRP4, MRP5 are expressed in macrophages (Table 1-2).

Each of these efflux transporters belongs to the ATP binding cassette family of efflux transporters (ABC). There are forty nine currently known members of the ABC family, most of which are approximately 1500 AA in size and comprised of two equal or unequal halves, contain one or two binding pockets, possess a membrane spanning domain and a transmembrane domain, and efflux substrate via an ATP dependent mechanism (Figure 1-9). [1,5,18,19,33,43,70]

Substrate specificity for efflux transporters

A wide variety of structurally unrelated potentially cytotoxic compounds are substrates for the ABC family of efflux transporters including antibiotics, immunosuppressive agents, chemotherapeutic agents, steroids, β-blockers, and HIV-1 protease inhibitors. [1,2,5,19,20,22,43,56,57,60,65,68,70,70,77,81,106]

Interaction between substrate and efflux transporter

There is no shuttle mechanism for substrate delivery to binding pocket(s) of efflux transporters. Most ABC efflux transporters possess two binding pockets for substrate, which increases the diversity of the transporter for substrate. Although two binding pockets exist, the conformation dictates that only one binding pocket may be occupied by substrate at one time. Thus, although two binding pockets exist, the efflux transporter may only efflux one substrate at a time. Substrate that is in close physical proximity to the binding pocket will be effluxed, whereas substrate that is physically distant from the transporter will remain intracellular. [1,5,18,19,70]

Activity state of efflux transporters

ABC efflux transporters remain in a conformationally inactive state until substrate binds to the substrate-binding pocket. This event confers a conformational change, which allows for activation of the efflux transporter, resulting in efflux of substrate from the cell. Activity state of the transporter is not dictated by any factor other than presence of substrate in binding pocket of

efflux transporter. Upon efflux of the substrate, the efflux transporter will again adopt the conformationally inactive state until another substrate binds to the binding pocket.[1,5,18,19,70]

Activity and expression of efflux transporters when substrate is present

In all cases, presence of PI activates the efflux transporter, allowing for efflux of PI via the ATP dependent mechanism, but does not increase expression levels of the transporter (with the exception of NFV). Of the ten PI, NFV is the only known PI that can not only activate the efflux transporter, but increase efflux transporter expression. Depending on the substrate, increase in efflux transporter expression may occur, as is the case with some NRTI. This finding has been demonstrated in multiple HIV-1 target cells including primary lymphocytes, macrophages, and T cell lines. [1,18,19,21,42,44,65,70,71,77,81, 93]

Presence of multiple substrates

Multiple agents are substrates for ABC efflux transporters. In HIV-1 infection, combination ART dictates the concomitant administration of multiple drugs. In an HIV-1 infected individual, multiple drugs may compete for binding to efflux transporters, and the presence of drugs with higher affinity for the efflux transporter-binding pocket can affect intracellular drug levels, where affinity is defined as ability of Drug A to bind to efflux transporter, conferring preferential efflux, when both Drug A and Drug B are present. When a drug with higher affinity is present with a drug of lower affinity, the higher affinity drug competitively inhibits the lower affinity drug. The higher affinity drug will be preferentially effluxed from the cell, and the lower affinity drug will remain largely intracellular. In this way, presence of multiple drugs in a system directly affects intracellular drug levels and ability of drug to be effluxed. [1,5,18,19,70]

Route of orally administered HIV-1 Protease Inhibitors

Protease Inhibitors are administered orally, and are highly metabolized by cytochromes, most notably CYP-450a and CYP-450b in the intestinal lumen [1,4,37,106]. The drug that is not metabolized traverses the intestinal lumen and circulates in the periphery, where the PIs are highly bound to plasma proteins (lowest percent bound = 60 % for IDV, highest percent bound = 98 % for Nelfinavir [NFV]). Of the PI that is free (non-plasma protein bound) to traverse the lipid bilayer and enter cells, lipophilicity dictates whether passive diffusion occurs, or whether entry must occur via active transport [1,4,37,106]. Upon entry into cells, PIs may bind to intracellular proteins, or, depending on efflux transporter expression (PIs are substrates for ATP-Binding-Cassette Family of efflux transporters), become rapidly effluxed from cells (Figure 1-8). PIs that are effluxed may again enter cells either by passive diffusion or active transport. [1,5,18,19,70] Metabolism, plasma protein binding, and efflux of PI present obstacles in treating HIV-1 infection. [1,2,4,5,6,9,18,19,35,43-44,57,60,70,71,81,84,85,94,106,116,117] Identifying mechanisms for decreased bioavailability of ART, and PIs in particular, can lead to design of novel therapeutics to increase intracellular concentrations of drug.

Impact of MDR1 genetic polymorphisms on intracellular bioavailability of PI

Many studies have been conducted to elucidate the relationship between efflux transporter expression, bioavailability of PI, and ability to control HIV-1 replication in infected patients. [6,9,12,20,39-41,44,57,58,61,71,72,77] Conflicting results have been reported in the literature, often as a function of differences in cohorts or cell type studied. Some studies report that in HIV-infected patients, IC_{50} of PI is inversely correlated with MDR-1 gene overexpression in lymphocytes and that undetectable viral loads are associated with the use of low-dose RTV (as a boost) [1,20,40]. Other studies report the contrary and state that there is a minimal effect of MDR1 and CYP3A5 genetic polymorphisms on the pharmacokinetics of IDV in HIV-infected patients, but do not

address impact of other PI either alone or in concert with RTV [104]. The data to date are unable to consistently draw a direct link between MDR1 genetic polymorphisms and intracellular bioavailability of drug, although few studies overlap in methods applied, reducing ability to directly compare results between studies.

Mechanisms to Study Intracellular Bioavailability of ART

Multiple mechanisms are routinely employed to study the intracellular bioavailability of ART, in particular HIV-1 PI, either directly or indirectly (Table 1-3). [1,19-22,30,41-44,56,57,59,60,63,65,66,68,71,73,77,83,93,97,98,106,107,112]

HPLC and mass spectrometry

High performance liquid chromatography coupled with mass spectrometry provides a direct assessment of intracellular drug available. This method is applied after methanol extraction of intracellular fraction of drug. Limitations for this method include total cell number required to observe intracellular PI levels above the limit of detection for the assay, and inability to observe intracellular fraction of drug within subsets of cells without prior sorting of subpopulations (Table 1-3).

Radio labeled PI

Use of radio labeled PI is employed to discover intracellular PI levels within cells. Radio labeled forms of PI are available for most PI, and intracellular PI can be observed within the population of cells treated with the radio labeled drug by detecting radioactivity of the sample. Limitations of this method include inability to concomitantly observe PI levels in cells while assessing receptor expression profiles, thus precluding this method from assessing intracellular PI levels within subsets of cells without prior sorting of subpopulations (Table 1-3).

Rhodmaine-123

Rhodamine-123 (Rh-123) is a fluorescent dye that can be detected on FL1 via flow cytometry. Hofmann, *et al* [68], first reported this method in 1992. The group first established a concert between Rh-123 intracellular accumulation and p-gp expression using cell lines that were positive and null for p-gp. The group assessed Rh-123 accumulation in cells isolated from patients suffering from acute myeloid leukemia (AML), a cell type known to have significantly elevated levels of p-gp, both in the presence and absence of a specific p-gp inhibitor. For cells without inhibitor, Rh-123 efflux was rapid, whereas in the presence of inhibitor, Rh-123 accumulated intracellularly and did not efflux (Figure 1-9).

To confirm the hypothesis that Rh-123 accumulation relates to p-gp activity and expression, single color flow cytometry was performed using a specific flurochrome conjugated monoclonal antibody against p-gp, MRK-16, in the AML cell lines (which rapidly effluxed Rh-123) and cell lines null for p-gp (which did not efflux Rh-123) Analysis confirmed that p-gp levels were high in the cells that rapidly efflux Rh-123, but were absent in alternate cell lines null for p-gp [68].

Shortcomings of this method are that this method provides indirect assessment of p-gp expression, instead observing p-gp activity. P-gp activity correlates with p-gp expression in most cases. In a system where p-gp expression levels may be altered by presence of substrate (for example, NFV), this method would not be ideal, and would require follow up with specific anti-p-gp monoclonal antibodies, western blot analysis, RT-PCR, or use of p-gp+/- cell lines to directly assess expression and modulation of p-gp by PI. [1,19-22,30,41 44,56,57,59,60,63,65,66,68,71,73,77,84,93,97,98,106,107,112] Comprehensive discussion regarding shortcomings, pitfalls, and alternative approaches to Rh-123 assay appears in the discussion section of this thesis, as it relates directly to the data reported in the thesis.

Many studies have been performed to study efflux transporter expression within subsets of cells. Various combinations of the assays described above (Rh-123, p-gp+/- cell lines, RT-PCR, western blot, flow cytometry) have been employed. A summary of these data and methods employed is as follows: [1,19-22,30,41-44,56,57,59,60,63,65,66,68,71,73,77,84,93,97,98,106,107,112]

Staining for efflux transporter expression

Another commonly used method for indirect assessment of bioavailability of ART is the indirect measure of efflux transporter expression via flurochrome-conjugated monoclonal antibodies. There are antibodies for all known efflux transporters for which PI are substrates, presenting a diverse method to assess six efflux transporters simultaneously within a target cell population via flow cytometry. This method can also be employed with receptor expression staining, allowing for concomitant determination of efflux transporter expression within subsets of cells (Table 1-3).

Shortcomings of this approach include the fact that although this is a direct measure of efflux transporter expression, it is an indirect method for determining intracellular drug levels. This method must be performed in tandem with other experiments, which can validate and support the results from this assay. Until recently, many of the antibodies commercially available were limited, thus truncating the ability to perform complex experiments to observe many parameters simultaneously.

Western blot for efflux transporters

Western blot analysis can be performed with antibodies specific for efflux transporters on cellular lysates obtained from PI or drug treated cells. In this way, direct assessment of efflux transporter expression as a function of time and exposure to PI can be observed (Table 1-3).

Use of efflux transporter +/- cell lines

Cell lines that are high p-gp expressers as determined from flow cytometry direct stain, Rh-123 efflux, casein efflux, western blot, RT-PCR or assessment of intracellular concentration of drugs that are substrates for p-gp but not other efflux transporters provide an excellent model to study effect of PI on p-gp activity and expression. Tumorogenic cell lines frequently over express efflux transporters as a mechanism of resistance to chemotherapeutic agents, which are substrates for p-gp or other efflux transporters. CEM-MDR cells, which express 30-fold more p-gp than CEM cells, and CEM-MRP cells, which express fivefold more MRP1 protein than CEM cells present a model to specifically study p-gp and MRP1.

Another mechanism of obtaining a cell line expressing high levels of efflux transporters is transfection of a cell line with MDR1 gene. L-MDR1 cells are a human epithelial kidney cell line transfected with expression vector expressing MDR1 cDNA, and P388/dx cells are a monocytic leukemia cell line that express high levels of p-gp. Both cell lines are commonly employed to assess direct modulation of efflux transporter activity and expression by PI and other drugs (Table 1-3).

RT-PCR for Efflux Transporters

Reverse Transcriptase-PCR provides a direct mechanism to observe modulation of mRNA transcripts by presence of substrate. Transcript levels before, during, and after addition of PI can be observed in a system comprised of cells expressing p-gp, allowing for direct assessment of efflux transporter expression (Table 1-3).

Studies Observing Relationship Between PI, Efflux Transporters, and Subsets of HIV-1 Target Cells

Many studies have been performed to study one or more aspects of efflux transporter expression and activity and its relationship to PI and subsets of HIV-1 target cells. The impact of

these data relative the findings of this thesis are addressed in the discussion. A summary of studies encompassing these data are as follows: [1,19-22,30,41-44,56,57,59,60,63,65,66,68,71,73,77,84,93,97,98,106,107,112]

Studies assessing p-gp mediated efflux of PI

Many studies have been performed to study the interaction between PI and efflux transporter activity and expression. Results are diverse and appear below. [1,19-22,30,41-44,56,57,59,60,63,65,66,68,71,73,77,84,93,97,98,106,107,112]

- PI inhibit Rh-123 efflux
- PI are substrates for p-gp (using Rh-123 assay)
- There is differential p-gp activity within total PBMC population
- There is differential p-gp expression within subsets of lymphocytes (assessed with direct stain for efflux transporters in concert with receptor expression stain)
- NFV but not other PI increases p-gp expression *in vitro* but not *in vivo*
- Increased expression and activity of p-gp is not related to intracellular HIV-1 RNA and DNA levels
- Hierarchy for lipophilicity: NFV > SQV > RTV > IDV (in PBMC assessed *in vitro*)
- Hierarchy for intracellular accumulation of PI: NFV > SQV > RTV > IDV (in PBMC assessed *in vitro*)
- PI interfere with p-gp function in targets of HIV-1 infection within the lymphocyte population
- ABC efflux transporters p-gp and MRP1 limit the intracellular bioavailability of IDV in macrophages *(in vitro)*

Studies using Rh-123 to assess p-gp activity in lymphocyte subsets

Upon establishment by Hofmann, *et al* that Rh-123 is a substrate for p-gp [68], Rh-123 has been employed by many groups to assess p-gp activity. The impact of these data relative the findings of this thesis are addressed in the discussion.

Schuitemaker *et al* sought to determine if p-gp expression or activity in HIV-1 target cells within the lymphocyte population correlates with intracellular viral RNA or DNA [89.] To answer this question, they studied Rh-123 levels in CD4+ T lymphocytes cells isolated from HIV-1 infected patients that were subsequently gated on CD45RO. The CD45RO cells were gated to observe two distinct populations: RO+/Rh-123 dim (high p-gp activity) and RO-/Rh-123 bright (low p-gp activity). The group determined that intracellular HIV-1 RNA and DNA was significantly lower in cells with high-p-gp activity, thus concluding that the potential efflux function of p-gp on PIs may be clinically less relevant than the effect of p-gp on intracellular HIV-1 replication.

Gastl, *et al* [73] assessed Rh-123 activity within lymphocyte subsets by gating on CD4, CD8, CD45RA and CD45RO, or on CD16/CD56 (NK cells). The group determined that CD4+/CD45RA+ cells display higher p-gp activity than CD4+/CD45RO+ cells.

Benedetti *et a l* [112] assessed p-gp activity with Rh-123 assay and direct p-gp expression in subsets of PBMC gated on CD3, CD4, CD8, CD16 and CD56. The group determined that p-gp activity is highest in NK cells and that p-gp activity is significantly higher in CD3+/CD8+ T cells relative to CD3+CD4+ T cells, but that p-gp activity is not always a good correlate for p-gp expression.

Gupta *et al* [7] studied Rh-123 activity in CD4 and CD8 T cells isolated from HIV-1 infected patients. The group determined that p-gp activity is low in chronically infected HIV-1 infected individuals with viral loads that predict disease progression. The group also stated that the study population would need to be increased to confirm these results.

Impact of ART Independent of Antiviral Effect

Little is known about the effect on the cellular level of entry inhibitors independent of their antiviral effect, and no potentially beneficial effects on the immune system have been

clearly mapped to T-20 or Selzentry treatment. Both NRTI and NNRTI have been implicated in modulation of host cell functions independent of their antiviral effect, most notably modulation efflux transporter function, activity and expression, and modulation of proteasome function [20,88,89].

Extensive literature exists to demonstrate that PIs have an effect on cells independent of their antiviral effect.[2,4,12,15,21,23,38,44,52—60,62,78,79,87-91,102,102,113-115] Effects include the following:

- Inhibition of cell cycle progression via G1 block in primary PBMC and cell lines
- Restoration of levels of circulating levels of cytokines in HIV-1 infected patients to levels observed in healthy patients (Type 2 to Type 1 switch)
- Inhibition of apoptosis both *in vitro* and *ex vivo* for both infected and bystander cells
- Modulation of antigen presentation
- Inhibition of proliferation in lymphocytes
- Modulation of lipoprotein metabolism
- Modulation of dendritic cell maturation

In a significant percentage of children and adolescents receiving a PI containing regimen, immune reconstitution is achieved but not concomitant with decrease in viral burden (viral failure, immune success; VF/IS) (Figure 1-10), implicating a role for PI in immune reconstitution independent of its antiviral effect.

Immune reconstitution is highly favorable for patient prognosis and leads to delayed progression to AIDS. [29,34-36] This discordant response is not determined solely by host factors alone or simple genetic changes in the viral genome. [13, 23-27, 39, 43, 46, 61, 83] ART induced discordant responses are multifactorial and involve a complex interplay between phenotypic properties of the virus, ART effect and interaction with host cells, and the ability of the immune system to control viral replication in the presence of ART.[13, 23-27, 39, 43, 46, 60, 83] The effect of PI on

the immune response independent of its antiviral effect, and the implications of differential effect of PI within subsets of HIV-1 target cells and in other cells, constitutes a complex set of questions relating to therapy outcome and progression to AIDS.

Therapy Response Groups

Plasma virus levels and CD4 T cell counts are used to classify patient response to ART. Patients who exhibit sustained suppression of viral replication (viral success) and significant improvement in CD4 T cell counts (immune success) are classified as Viral Success Immune Success (VS/IS). This occurs in 25-30 % of children. Patients who fail to maintain suppression of viral replication (viral failure) and do not exhibit improvement in CD4 T cell counts (immune failure) are classified as Viral Failure Immune Failure (VFIF). This occurs in 15-20 % of pediatric patients. Patients who exhibit sustained reconstitution of T cell counts (immune success), despite transient viral suppression and subsequent rebound to levels that predict disease progression are classified as Viral Failure Immune Success (VF/IS). Discordant response (VF/IS) occurs in 25-40 % of children ad adolescents, and is maintained in adults in a variety of cohorts (Figure 1-10). [39, 100, 116,118]

The goal when treating HIV-infected patients is to delay progression to AIDS by controlling viral replication and preventing or reversing immune deficiency, and viral loads and T cell counts directly correlate with clinical disease progression[29,34,36]. For example, children who display discordant response exhibit lower clinical disease progression rates than those of control groups with similar viral levels who do not display immune reconstitution (implicating critical role of immune reconstitution in favorable clinical prognosis) [46,100,108]. In addition, chronic HIV infection and high viral loads globally impair immunity via multiple mechanisms such as CD4 T cell attrition by direct infection and apoptosis, HIV-induced uncontrolled activation of adaptive and innate immune response, impairment of thymic output, and evasion of

HIV-specific immunity. [23,34,36,29,53,54,103] Therefore, controlling viral replication while maintaining or restoring functional immunity remains sentinel in treating HIV-infected individuals.

Hypothesis

Previous studies in our laboratory determined that significantly more drug (188 fold for IDV and 141 fold for RTV) is required to inhibit viral replication for otherwise identical viruses targeted to R5 versus X4 expressing T-lymphocytes (Figure 1-.11).

The hypothesis of this thesis is that differential bioavailability, and specifically differences in efflux kinetics and peak intracellular PI levels in subsets of lymphocytes are responsible for requirement of significantly higher IC_{50} in the R5 subset of HIV-1 target cells.

Specific Aim 1a

Previous studies in our laboratory demonstrated that significantly more drug is required to inhibit viral replication for otherwise identical viruses targeted to R5 versus X4 T lymphocytes (Figure 1-11). To test the hypothesis that differences in efflux kinetics and peak intracellular PI levels in subsets of HIV-1 target cells within the lymphocyte population are responsible for these data, the optimal conditions at which to observe peak intracellular PI levels and efflux kinetics in subsets of CD4+ T lymphocytes were determined, using U937 promonocytic cell line, and total PBMC. Using U937 and PBMC provided a model to observe inter and intra-assay variability, as well as optimized conditions. This specific aim was addressed with the following questions:

- Published data demonstrates that peak intracellular PI levels in PBMC are conferred by 18 hours extracellular PI treatment at 37^{o}C. Can we recapitulate similar values in our laboratory?
- What is the inter-assay and intra-assay variability for this study?
- What are the optimal conditions to observe baseline intracellular PI levels above limit of detection in PBMC? (Variables include serum concentration in extracellular media and stimulation state of cells)

- What are the optimal conditions to observe efflux kinetics of PI in PBMC? (Variables include serum concentration in extracellular media, temperature, time, and stimulation state of cells)

Specific Aim 1b

Upon optimization of conditions at which to observe peak intracellular PI levels and efflux kinetics, the receptor expression profile for these PBMC was observed. This experiment was conducted to determine if the total number of R5 positive HIV-1 target cells within the lymphocyte population was high enough to sort, and employ peak intracellular PI and efflux kinetics studies as optimized above. To address this specific aim, the following questions were posed:

- What is the receptor expression profile for subsets of T-lymphocytes for conditions that are optimal to observe intracellular PI levels in PBMC?
- The limiting factor is the R5 subset of HIV-1 target cells within the lymphocyte population. Will there be enough of this lymphocyte subpopulation under the optimized conditions?

Specific Aim 1c

It was determined that the conditions at which peak intracellular PI levels and efflux kinetics can be observed results in a total number of R5 positive HIV-1 target cells within the lymphocyte population which may be too low to sort and subsequently employ efflux and peak intracellular PI studies. Alternative approach was employed using a Rhodamine-123 assay (Rh-123) to observe p-gp activity within subsets of CD4+ T lymphocytes. A specific question was posed as a means to determine an alternative approach, ultimately culminating in the use of Rh-123 collaborative study, as follows:

- If the cell number is too low to obtain enough of the R5 subset, what alternative approaches can be employed to test the hypothesis?

Significance

Suboptimal levels of ART is an ongoing problem in treating HIV-1 infection and presents obstacles including emergence of resistant mutations and clearance of virus from infected cells with low bioavailability. [1,6,9,10,21,40,43,76,77,81] Understanding dynamics of virus/host/drug interaction and identifying mechanisms for decreased bioavailability of ART can lead to design of novel therapeutics to increase intracellular concentrations of drug.

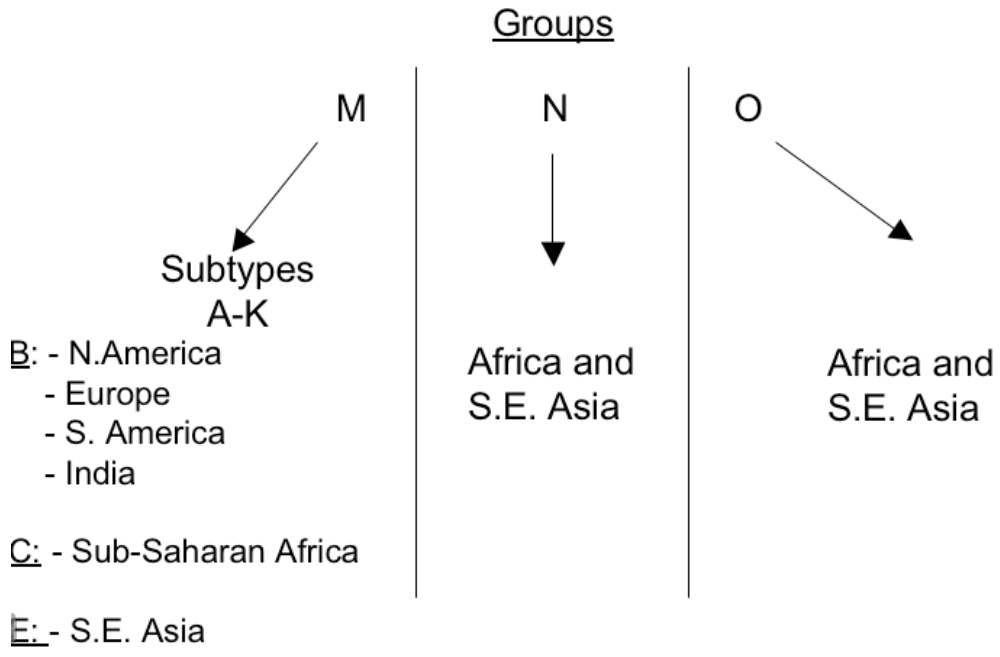


Figure 1-1. Global distribution of HIV-1 by groups, subtypes, and recombinant forms, organized by geographical location. Group M (major) contains subtypes A-K. Two new groups, N (new) and O (outliner), have been identified in Africa and Eastern Europe [29,92].

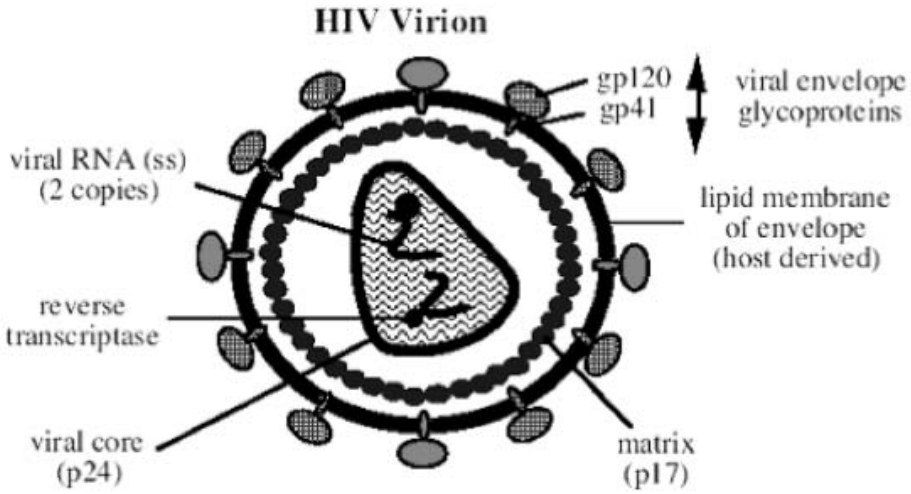


Figure 1-2. Structure of HIV-1 virion. HIV-1 is comprised of a nucleocapsid core, which contains two copies of the viral genome, core proteins, and reverse transcriptase (RT). The viral envelope, surrounds the core and consists of transmembrane gp41), and the surface subunit (gp120) [29, 45,92,100].

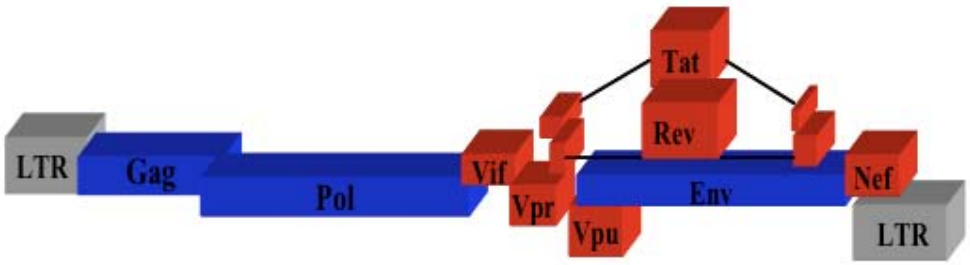


Figure 1-3. Genomic organization of HIV-1. Two identical long terminal repeats (LTR; purple) flank the viral genome, which includes the main genes *gag*, *pol*, and *env* (blue), and accessory genes *vif*, *vpr*, *vpu*, *tat*, *rev*, and *nef* (red) [29, 45,92,100]. This figure was obtained from Goodenow Lab Share drive and is unpublished.

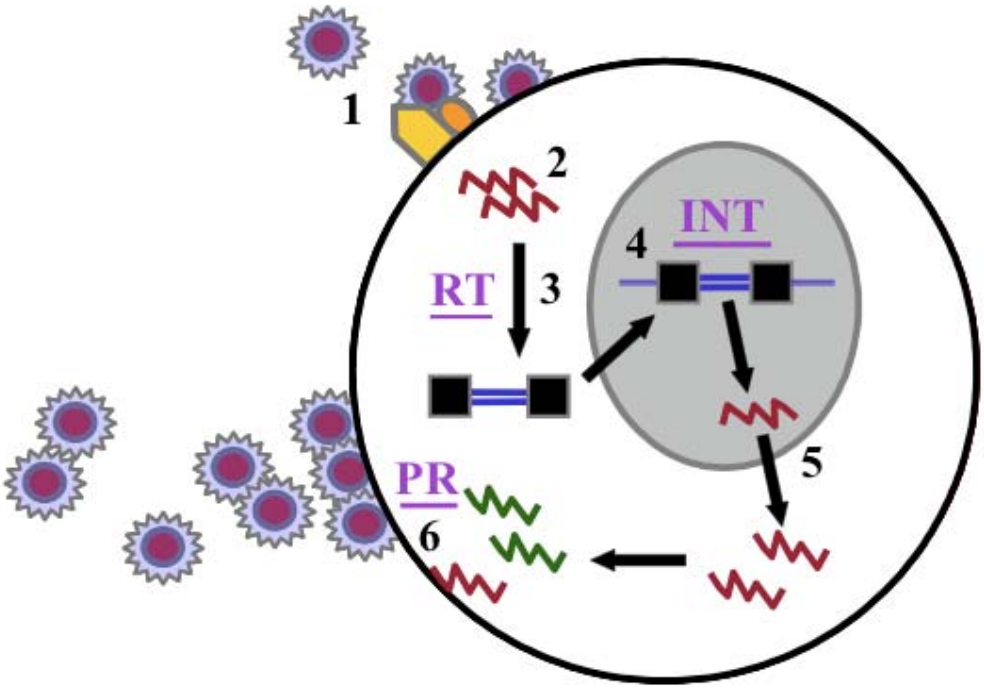


Figure 1-4. HIV-1 Life Cycle. Attachment of HIV-1 to host cell via CD4 and coreceptor (1); fusion; release of viral RNA into the cytoplasm of the host cell (2); Reverse Transcription (3); Integration (4); translation of gene products (5); budding and assembly at plasma membrane and processing by HIV-1 PR producing a mature virion (6) [27, 29, 45,92,100]. This figure was obtained from Goodenow Lab Share drive and is unpublished.

Table 1-1. Summary of HIV-1 coreceptor use and tropism. Tropism and coreceptor phenotype designation of virus appears in left column. Target cell coreceptor use appears in middle column. Target cell tropism appears in the far right column[8, 14, 17,29,32,45, 48, 49, 80]. This table was created by Christina Gavegnano in powerpoint and is not published.

Tropism and Coreceptor Phenotype Designation	Target Cell Coreceptor Use	Target Cell Tropism
L-R5	CCR5	Primary T Lymphocyte
M-R5	CCR5 CCR5	Primary Macrophage Primary T Lymphocyte
D-R5X4	CXCR4 CCR5 CXCR4 CXCR4	Primary T Lymphocyte Primary Macrophage Primary Macrophage CD4+ T cell line
DX4	CXCR4 CXCR4	Primary Macrophage CD4+ T cell line
TX4	CXCR4 CXCR4	Primary T Lymphocyte CD4+ T cell line

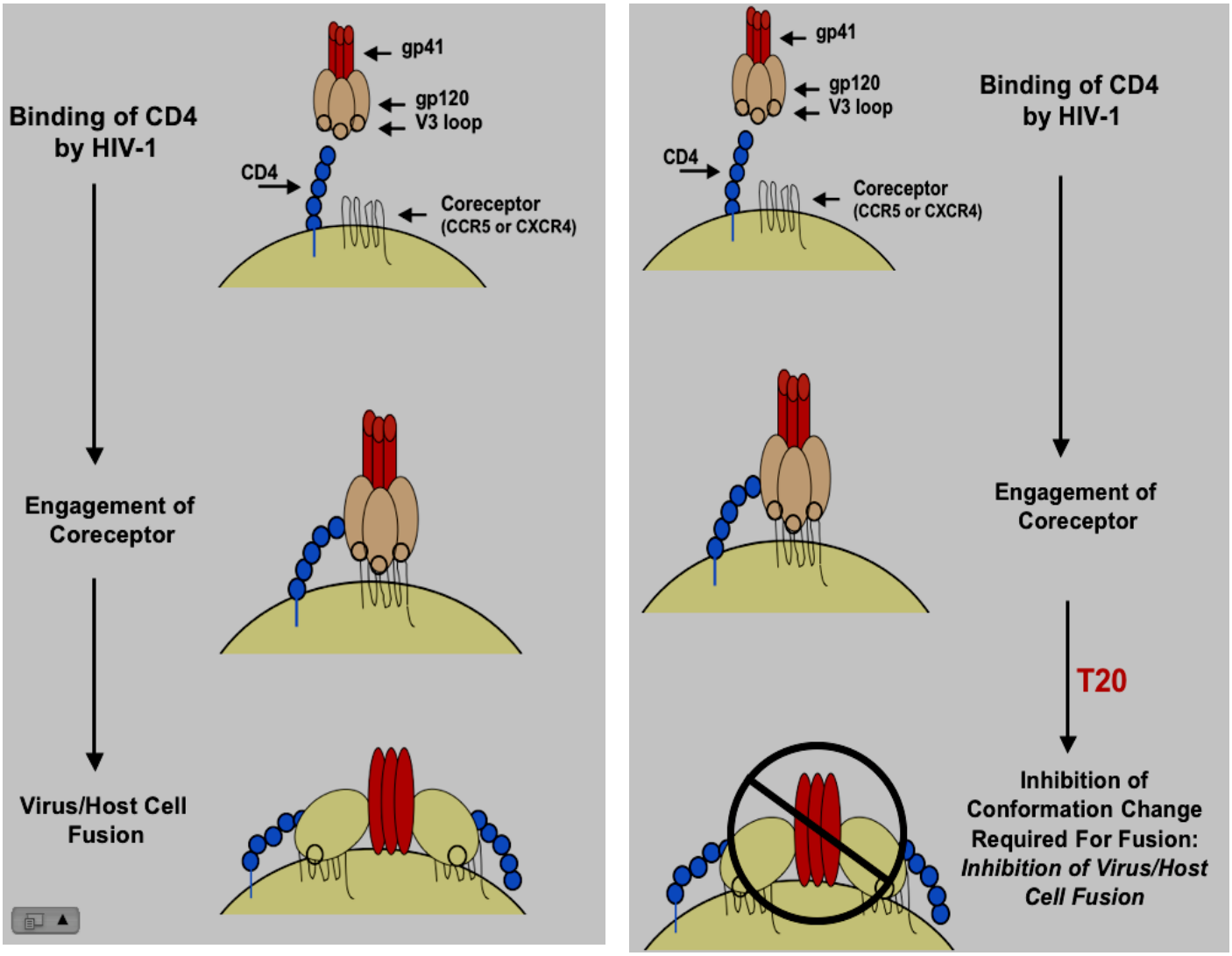


Figure 1-5. Mechanism of action for HIV-1 fusion inhibitor T20. Without fusion inhibitor, virus engages CD4 receptor followed by coreceptor and virus/host cell fusion is subsequently mediated (left panel). T20 prevents formation of intermediate hairpin structure required for fusion, thus preventing fusion of virus with host cell membrane (right panel) [11, 29,111,116]. This figure was created from scratch in powerpoint by Christina Gavegnano and is not published.

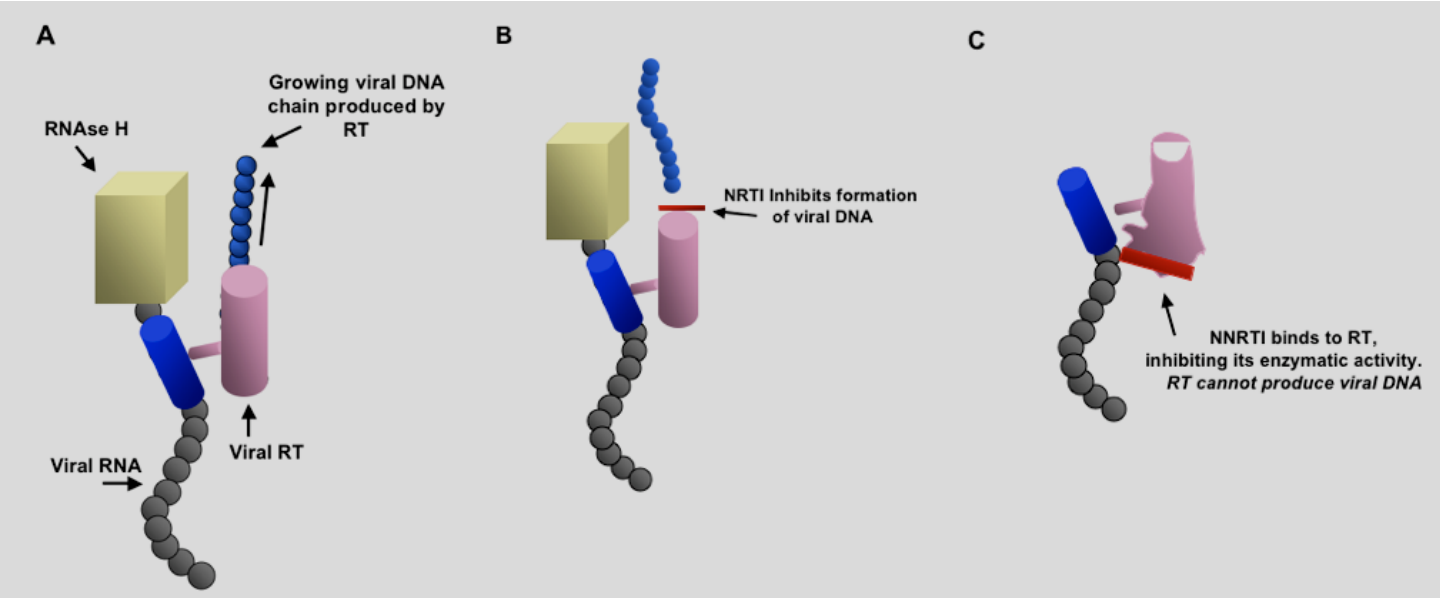


Figure 1-6. Mechanism of action for HIV-1 Reverse Transcriptase Inhibitors. NRTI compete with endogenous nucleotides for incorporation into growing viral DNA strand, but lack a 3´hydroxyl terminus. (B) Incorporation of NRTI into growing DNA strand results in termination of DNA strand (the next phosphodiester bond is not formed). (C) NNRTI bind directly to RT, inhibiting its enzymatic activity. [29,100,116] This figure was created from scratch in powerpoint by Christina Gavegnano and is not published.

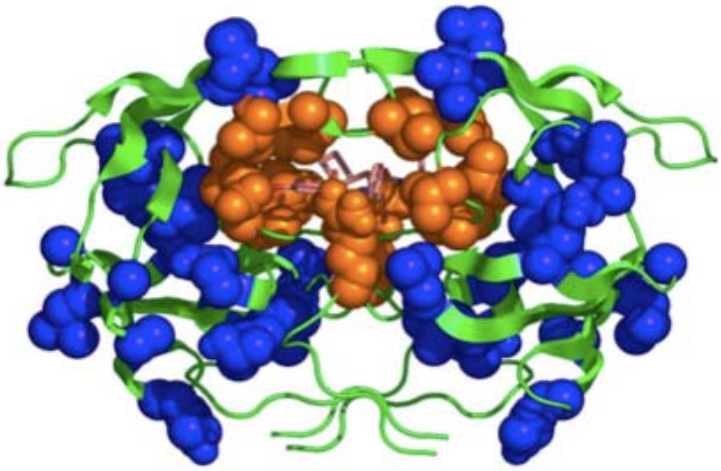


Figure 1-7. Structure of HIV-1 Protease with Protease Inhibitor bound. PI target HIV-1 PR, an aspartic protease comprised of two identical 99 amino acid monomers. PI are competitive inhibitors, which compete for binding in the active site with the natural substrate. Once bound, PI cannot be cleaved, resulting in inactivation of the enzyme. Backbone (green ribbon), active sites (red), and non active sites (blue). PI is bound to the active site (pink) [25-27, 29,100,116]. This figure was obtained from the Goodenow lab share drive and is unpublished.

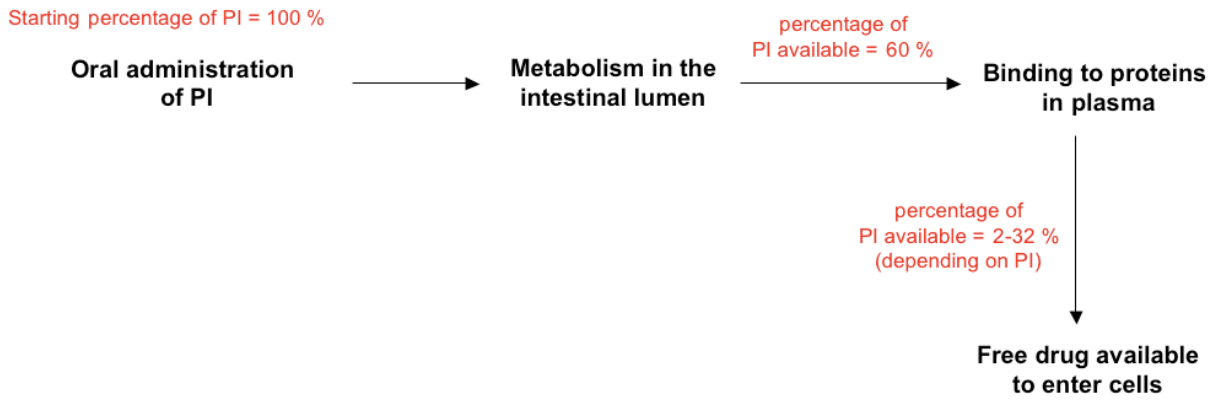


Figure 1-8. Route of orally administered HIV-1 Protease Inhibitors. HIV-1 PI are administered orally and are highly metabolized in the intestinal lumen by cytochromes. Upon entry into the circulating periphery, PI are highly bound to plasma proteins, which inhibits their ability to enter cells. Depending on the PI, 2-32 % of initial dose of PI is available to enter cells. [1,2,4,5,6,9,12,18-22,35,37,40,42,44,51,56,57,60,63-66,68,70-73,76,77,81,93,94,98,100,104,106,107,109,112] This figure was created from scratch by Christina Gavegnano using powerpoint and is unpublished.

Table 1-2. Efflux transporters for which PI are substrates and their expression in HIV-1 target cells. P-glycoprotein (p-gp/MDR1), MRP1, MRP2, MRP5 and BCRP are expressed in lymphocytes. P-gp, MRP1, MRP4, MRP5 are expressed in macrophages.[1,2,4,5,6,9,12,18-22,35,37,40,42,44,51,56,57,60,63-66,68,70-73,76,77,81,93,94,98,100,104,106,107,109,112] This table was created from scratch in powerpoint by Christina Gavegnano and is unpublished.

P-glycoprotein (MDR1)	MRP1	MRP2	MRP4	MRP5	BCRP
Lymphocytes Macrophages	Lymphocytes Macrophages	Lymphocytes	Macrophages	Lymphocytes Macrophages	Lymphocytes

Table 1-3. Methods to assess efflux transporter expression or activity and intracellular bioavailability of drug. Assays are listed in left column. Whether the assay is a direct or indirect measure of efflux transporter expression or intracellular drug levels is listed in middle and right columns, respectively. This figure was created from scratch by Christina Gavegnano in powerpoint and is not published. [1,19-22,30,41-44,56,57,59,60,63,65,66,68,71,73,77,84,93,97,98,106,107,112]

Assay	**Direct or Indirect Measure of Efflux Transporter Expression?**	**Direct or Indirect Measure of Intracellular Drug Levels?**
HPLC/Mass Spec.	Indirect	Direct
Radio Labeled Drug	Indirect	Direct
Rh-123	Indirect	Indirect
Fluorescent Stain for Efflux Transporter	Direct	Indirect
Western Blot for Efflux Transporters	Direct	Indirect
Efflux Transporter +/- Cell Lines	Direct or Indirect *(dictated by assay cells are subjected to)*	Direct or Indirect *(dictated by assay cells are subjected to)*
RT-PCR	Direct	Indirect

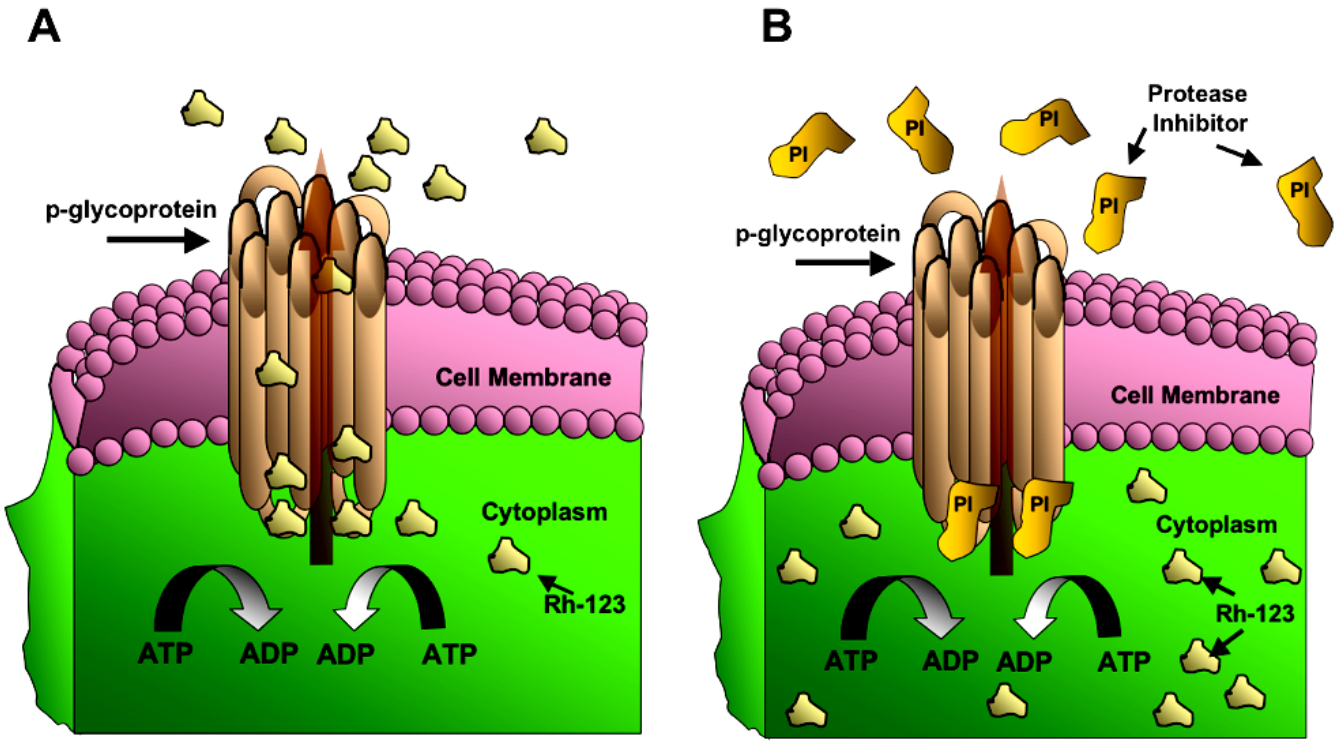


Figure 1-9. Effect of PI on mechanism of Rhodamine-123. (A) Rh-123 passively diffuses into cells and can be effluxed by p-gp. (B) PI bind with greater affinity to p-gp than Rh-123, therefore PI are preferentially effluxed by p-gp, and Rh-123 accumulates inside the cells in a ratio that is proportional to the amount of PI that has been effluxed by p-gp. [68] This figure was created from scratch by Christina Gavegnano in powerpoint and is not published.

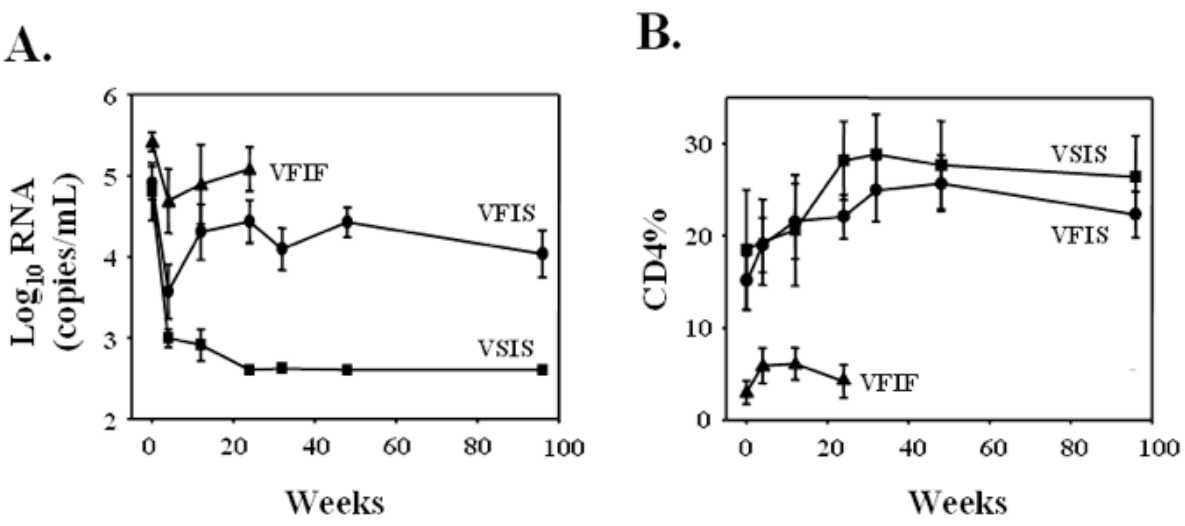


Figure 1-10. Viral and immune responses to HIV-1 antiretroviral therapy. Median $\log_{10}$ viral RNA copies/mL (Y axis) versus weeks (X axis) (A). Median CD4 T-cell counts per microliter (Y axis) versus weeks (X axis)(B). Upon ART treatment, VFIF viral loads do not decrease and CD4 T-cell counts do not increase; VSIS viral loads decrease and CD4-T cell counts rebound; VFIS viral loads transiently decrease but rebound to levels similar to pretherapy and CD4 T-cell counts rebound. This figure was created by Sarah K. Ho and is unpublished. [39,100, 116,118]

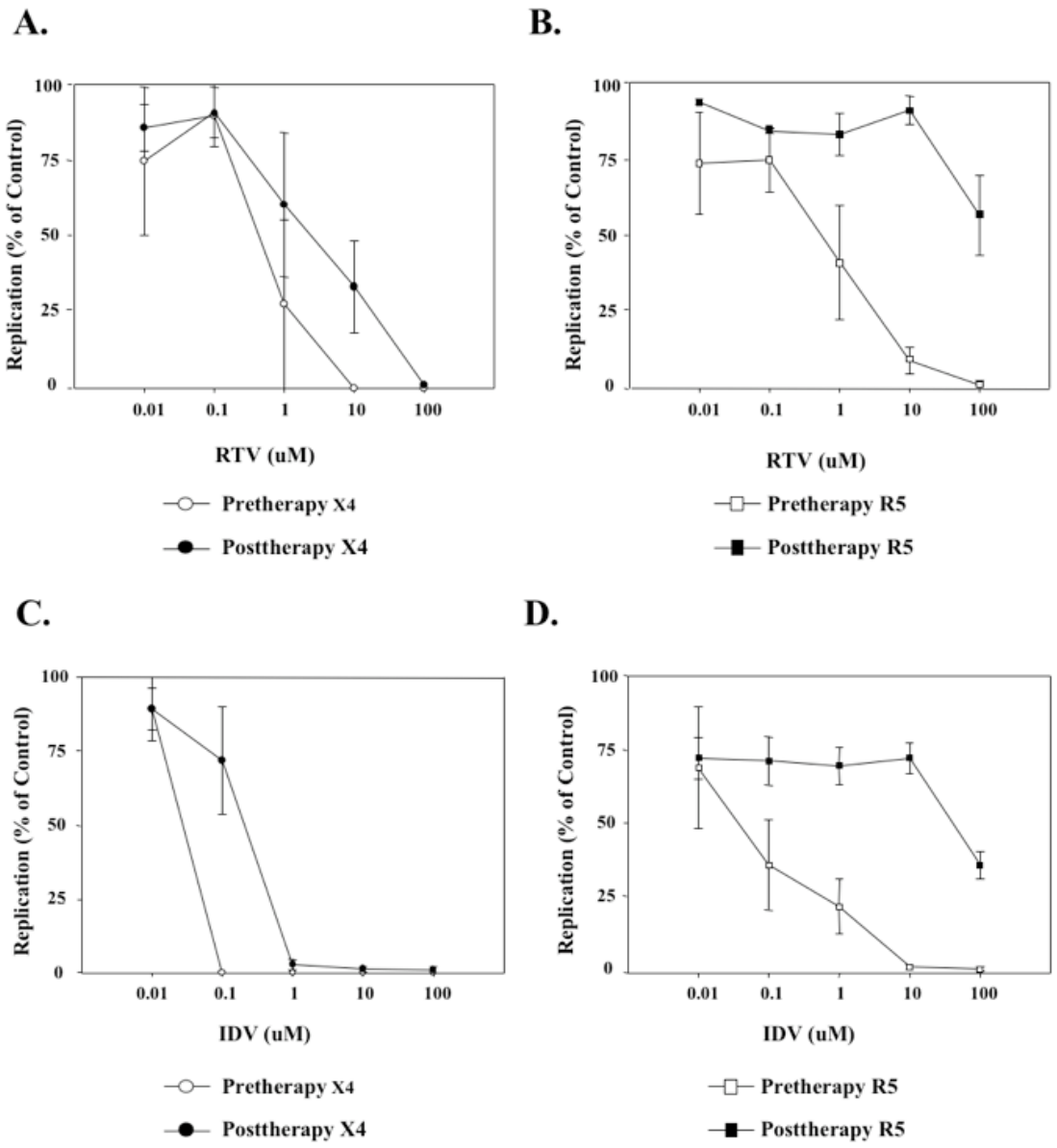


Figure 1-11. Significantly more drug is required to inhibit HIV-1 replication for viruses targeted to otherwise identical viruses targeted to R5 versus X4 expressing T-lymphocytes. IC_{50} curve taken from day 8 of 10 day infection for LAI (TX4 or MR5 envelope as indicated) pretherapy or posttherapy variants in the presence of RTV (panel A, B) or IDV (panel C, D) in CXCR4 or CCR5 expressing PBMC. More drug is required to inhibit X4 posttherapy relative to X4 pretherapy variant (A, filled circles, open circles, respectively), and more drug is required to inhibit posttherapy R5 variant relative to R5 pretherapy variant (B, filled squares, open squares, respectively). More drug is required to inhibit X4 posttherapy relative to X4 pretherapy variant (C, filled circles, open circles, respectively), and more drug is required to inhibit posttherapy R5 variant relative to R5 pretherapy variant (D, filled squares, open squares, respectively). Data represent mean and SEM of three independent donors. This figure was created by Sarah K. Ho and is unpublished.

CHAPTER 2
MATERIALS AND METHODS

Determination of Peak Intracellular PI Levels

This experiment was performed to answer part of specific aim 1a, question 1. PBMC for this study were prepared by Ficoll (Histopaque 1077; Sigma Diagnostics, St. Louis, Mo.) gradient density centrifugation from leukopack obtained from LifeSouth/Civitan Blood (Gainesville, FL). PBMC were resuspended at a concentration of 10^6 cells/mL and stimulated for 72 hr at 37^{o}C, 5 % CO_2, with 10 mg/mL phyto-hemagglutinin (PHA) (Sigma Diagnostics) in RPMI 1640 medium (Gibco/Invitrogen, Grand Island, N.Y.) supplemented with 30 U/mL human interleukin-2 (Boehringer Mannheim), 2 mM L-glutamine and 100 U of penicillin per mL, 100 g of streptomycin (Gibco/Invitrogen) per mL, and 10 % heat- inactivated fetal calf serum (Gibco/Invitrogen).

Three days post-stimulation, 15 x 10^6 cells were incubated with 1, 10, and 100 μM of the protease inhibitors IDV and RTV (obtained from NIH AIDS Reagent and Reference Program, Bethesda, MD) in duplicate in T-25 flasks (Starstedt, St. Louis, MO) (at 37°C, 5 % CO_2). At 3, 18, and 48 hours post-treatment, 5 x 10^6 cells were removed from each flask for drug extraction. The cells were aliquoted into 15 mL conical tubes (Starstedt) washed 3 times at 4^{o}C in 5 mL of ice cold 1 X Dulbecco's Phosphate buffered saline (PBS, Gibco/Invitrogen), and centrifuged (Beckman Coulter, Fullerton, CA) at 700 x *g* for 6 minutes at 4°C. Following the third wash, the cells were counted and extracted in 70% CH_3OH overnight at 4°C rotating, with a final concentration of

1x10^6 c/mL.

The following day, the samples were spun at 700 x *g* for 6 minutes at 4°C, and the supernatant fraction containing the intracellular drug, was transferred to a 1.8 mL cryovial and stored at -20°C until shipped to collaborators for mass spectroscopy analysis. To minimize drug loss, all steps were performed within an hour at 4°C.

Liquid Chromatography (LC) followed by Mass Spectrometric analysis (for confirmation of LC readout) by Lane Bushman from collaborator Courtney Fletcher's laboratory (University of Colorado) was performed on CH_3OH extracts to determine intracellular [PI] in each sample. For LC, a methods internal standard (RTV or IDV analog) was added to each sample. Sample was then dried, resuspended in mobile phase (in a blank matrix of cellular lysate in CH_3OH), and injected into the system (Figure 2-1). The labeling/designation for all duplicate samples that were NOT forwarded to Colorado, both for these studies and all other intracellular PI studies is as follows: Date-concentration of extracellular drug-timepoint for efflux-temperature of efflux-cell type. For example, a sample obtained on January 1, 2005 treated with 1 μM RTV, allowed to efflux for 10 minutes at 4°C in PBMC would read as follows: 20050101-1μM RTV-4°C-10 minutes-PBMC. All samples were stored in 81 place boxes in 1.5 mL cryovials at -80°C at the completion of each experiment.

Efflux Studies: Intracellular Protease Inhibitor Experiments

This experiment was performed to answer part of specific aim 1a, question 2-4. PBMC were isolated from leukopack obtained from LifeSouth/Civitan (Gainesville, FL) as described previously, and either PHA stimulated (10 μg/mL PHA for 72 hours at 37°C) or immediately exposed to 1.0 μM or 10.0 μM RTV containing RPMI 1640 medium with 2.0 % or 10.0 % fetal calf serum supplemented with 2 mM L-glutamine and 100 U of penicillin per mL, 100 g of streptomycin per mL. Cells were resuspended at a concentration of 1.0 x 10^6 cells/mL for 18

hours at 37°C, 5 % CO_2. U937 promonocytic cell line (American Type Culture Collection, Manassas, VA) were treated analogously with the exception that U937 were not treated with PHA before exposure to RTV containing media. After 18 hr. exposure to drug, 5.0 x 10^6 cells were aliquoted into 15 mL conical tubes, centrifuged at 4°C at 700 x *g* for 6 minutes to remove PI containing media, and washed three times in ice cold 1X PBS (4°C, 700 x *g*) to remove residual drug. Intracellular levels of PI were determined at baseline (immediately after washings), 10, 20, 40, 60, and 120 minutes post washing at 4°C, 20°C, or 37°C. At each time point, cells were immediately centrifuged (4°C, 700 x *g*) to remove residual drug. Efflux was then determined over the time course of baseline (immediately after washings), 10, 20, 40, 60, and 120 minutes post washing at 4°C, 20°C, and 37°C. At each time point, cells were immediately centrifuged (4°C, 700 x *g*) for 6 minutes and resuspended in ice-cold 70% CH_3OH for 4 hours at 4°C. Collaborator Lane Bushman of Courtney Fletcher's laboratory performed liquid Chromatography (LC) followed by Mass Spectrometric analysis (for confirmation of LC readout) (University of Colorado) to determine intracellular [PI] in each sample (Figure 2-2). A positive control sample was prepared by spiking 1 mL of 70 % CH_3OH with RTV stock to a final concentration of 10 uM to confirm concentration of RTV stock used for the experiments. For LC, a methods internal standard (RTV analog) was added to each sample. Sample was then dried, resuspended in mobile phase (in a blank matrix of cellular lysate in CH_3OH), and injected into the system (Figure 2-2).

Concentration of ng/mL was converted to μM by first converting ng/mL into g/mL and dividing this value by the MW of the PI to obtain mol. To determine the intracellular concentration of drug per cell, the mol value was divided by the volume of 1.0 x 10^6 cells, which is the total number of cells in 1mL. This cell number to volume ratio (1.0 x 10^6 cells/mL)

corresponds to the concentration of cells in the sample where the intracellular PI was quantified. Therefore, using this value to determine μM readout yields an intracellular PI value that correlates to the concentration of drug in each cell (Figure 2-3). For U937 and PBMC, standard error of the mean (SEM) and standard deviations were calculated to determine intraassay and interassay variability, respectively (Sigma Stat 3.0).

Receptor Expression Profile Study in CD4+ T lymphocytes

This experiment was performed to answer part of specific aim 1b, questions 5 and 6. CD4+ lymphocytes were isolated from leukopack obtained from Lifesouth/Civitan (Gainesville, FL) using human CD4+ T cell enrichment cocktail (Stemcell Technology, Seattle, WA), which depleted cells expressing CD8, CD16, CD14 (monocytes), CD36, CD56, and CD66b), and enriched for CD4+ T lymphocytes (Figure 2-4). Cells were cultured at $1x10^6$ cells/mL in RPMI 1640 and resuspended in either 2 % or 10 % heat inactivated fetal calf serum containing media, with or without PHA stimulation, and supplemented with 2 mM L-glutamine and 100 U of penicillin per mL 100 g of streptomycin (Gibco/Invitrogen).

At timepoints of baseline (immediately after isolation), 1, 3, and 5 days, cells were aliquoted into 50 mL conical tubes (Starstedt), and centrifuged for 10 minutes at 1000 rpm, and residual media was removed.

Cells were suspended in FACS wash [phosphate-buffered saline (PBS) containing 10% fetal calf serum, 2% normal human AB serum (Sigma), and 0.02% sodium azide (Fisher Scientific)], and flurochrome conjugated monoclonal antibodies specific for CD4, CD14, CCR5, or CXCR4, or isotype controls for 30 minutes at 4^oC. Fluorescence-tagged monoclonal antibodies specific for CCR5 (CCR5-PE), CXCR4 (CXCR4-APC), CD14 (CD14-FITC), and CD4 (CD4-PerCP) were obtained from BD Pharmingen (San Diego, CA), as were isotype

matched control monoclonal antibodies. After incubation, cells were subjected to centrifugation for 8 minutes at 1000 rpm at room temperature with cold FACS wash, and fixed in 1% ice-cold paraformaldehyde (Sigma).

Coreceptor expression by subsets of CD4 T cells was observed via initial gate drawn around CD4+ population (R1). R1 was then assessed for CD14 expression on a plot of CD14 versus forward scatter. R1 was also assessed for either CXCR4 expression or CCR5 expression on a plot of coreceptor expression (either CXCR4 or CCR5) for the R1 population versus percent of cells. Gates for CD4+/CCR5+ and CD4+/CXCR4+ lymphocytes were established based on plot of isotype matched controls for either CXCR4 or CCR5. Data acquisition was performed using an FACS Vantage flow cytometer (Becton Dickinson) and FloJo Software, Version 7.0,Treestar, MD). Experiments were conducted in three independent donors, with technical duplicates per donor. Standard error of the mean (SEM) and standard deviation were determined using Sigma Stat 3.0.

Determining p-gp activity and Inhibition of Rhodamine-1, 2,3 (Rh-123) Efflux by PIs

Collaborator Matthew Morrow at University of South Florida, Sleasman laboratory, performed Rh-123 studies. This experiment was conducted to answer specific aim 1c, question 7. PBMC from fresh whole blood were isolated using Histopaque density gradient centrifugation as described previously. PBMC were cultured at $1x10^6$ cells/mL in complete RPMI-1640 media (10% fetal calf serum, 2 mM L-glutamine, 100U/mL penicillin, and 100 μg/mL streptomycin) were treated for 30 minutes at 37°C with 0.5 μg/mL Rh-123 (Molecular Probes – Invitrogen) either alone or in the presence of 10 μM R-Verapamil (Sigma), 10 μM (RTV), 10 μM (IDV), or 10 μM (NFV). After 30 minutes, the cells were transferred to fresh complete RPMI-1640 lacking Rh-123 and PI. The cells were cultured at 37°C in 5% CO_2 and samples were collected at 1 and 2.5 hours time points for analysis of intracellular levels of Rh-123 using flow cytometry.

Cells were washed with 1X PBS at 4^{o}C, and then stained for surface CD3 (CD3-), CD4 (CD4-), CD8 (CD8-), CCR5 (CCR5-), CD45RA (CD45RA-), and CD45RO (CD45RO-) using cold buffers to inhibit further Rh-123 efflux. All antibodies were obtained from BD Biosciences (San Jose, CA). Data acquisition was performed using an LSR-II flow cytometer (BD Biosciences), and data analysis was performed using FlowJo software, version 7 (Treestar, Portland, OR).

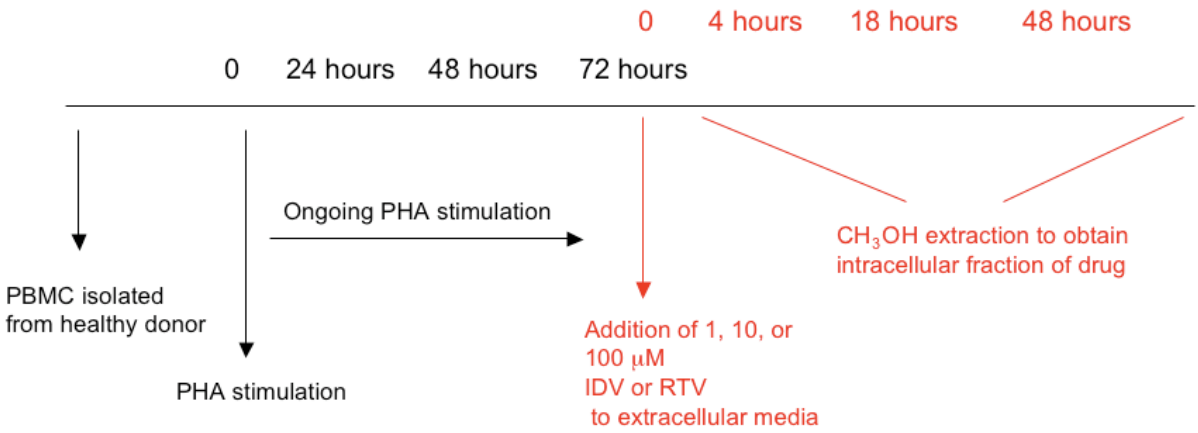


Figure 2-1. Experimental design for pilot peak intracellular Protease Inhibitor study. PBMC were isolated from healthy donor and immediately subjected to PHA stimulation for 72 hours. After 72 hours, 1, 10, or 100 µM IDV, RTV, or NFV was added to extracellular media, and cells were harvested at 4, 18, or 48 hours post addition of PI.

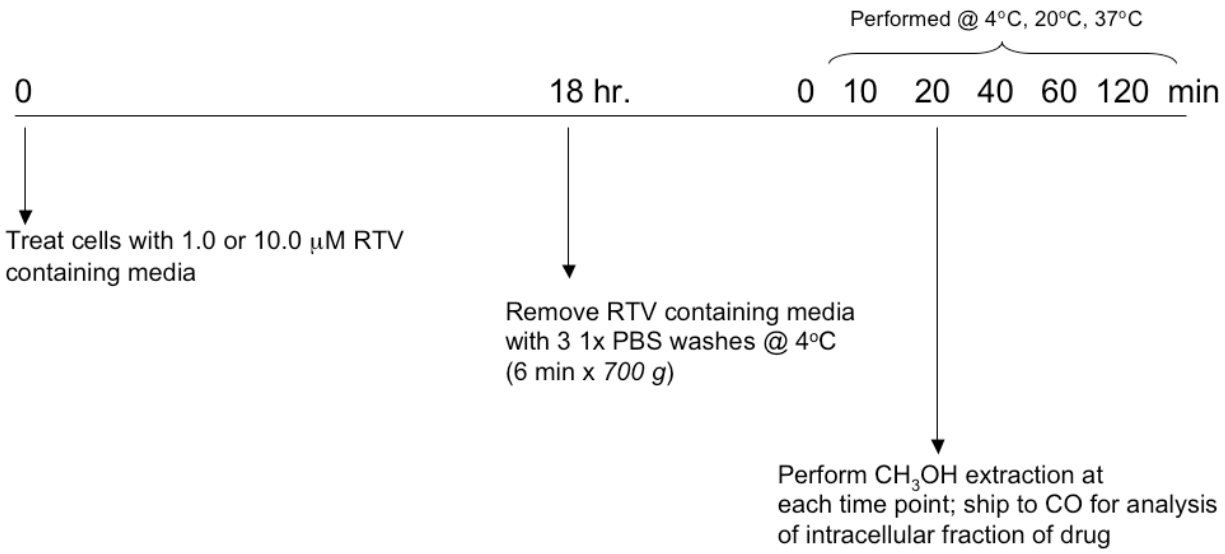


Figure 2-2. Experimental design for Protease Inhibitor efflux study. PBMC or U937 were treated with 1 or 10 μM RTV containing media for 24 hours prior to removal of RTV containing media. Methanol extraction was subsequently performed on cells to obtain intracellular fraction of drug.

Values are reported in ng/ml (as determined from HPLC and confirmed with mass.spectrometry)

18 hr. 1.0 uM RTV Tx = 1.4 ng/ml
1.4 ng = 1.4×10^{-9} g

g/MW = mol
MW RTV = 720.96

$$\frac{1.4 \times 10^{-9}\ \text{g}}{720.96} = 1.9 \times 10^{-12}\ \text{mol}$$

M = mol/L
For volume (L), the volume of 1.0×10^{6} cells (the amount of cells/ml) will be determined:
Volume of 1 PBMC = ~ 0.4 pL = 0.4×10^{-12} L = 4.0×10^{-13} L

$(1.0 \times 10^{6}\ \text{PBMC}) \times (4.0 \times 10^{-13}\ \text{L}) = 4.0 \times 10^{-7}\ \text{L}$

$$M = \frac{1.9 \times 10^{-12}\ \text{mol}}{4.0 \times 10^{-7}\ \text{L}} = 4.8 \times 10^{-6}\ M = \boxed{\textbf{4.8 uM}}$$

Figure 2-3. Sample calculation for conversion of ng/mL to µM for intracellular Protease Inhibitor experiments. Gram value was converted to mol, and mol value was converted to molarity using the equation mol/L, where L volume is determined by the volume of cells in the sample.

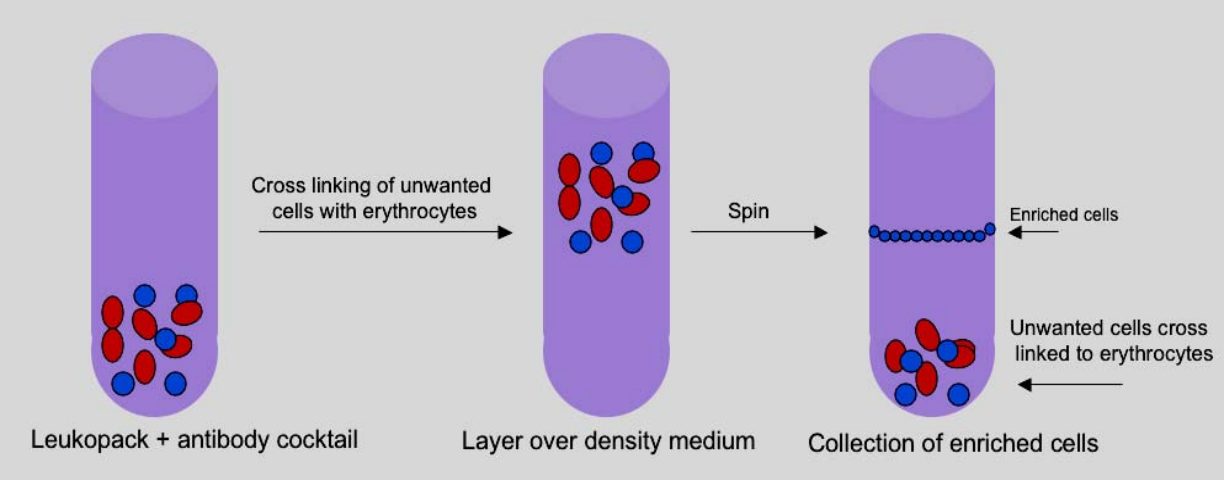


Figure 2-4. CD4+ T cell enrichment procedure. Leukopack is subjected to antibody cocktail containing antibodies specific for monocytes (CD14), and depletion of all cells that do not express CD4. The antibodies bind to the unwanted cells, and subsequently cross-link with the erythrocytes. Upon centrifugation, the unwanted cells are pulled to the bottom of the tube, whereas the enriched population of desired cells appears in a monolayer.

CHAPTER 3
RESULTS

Review of Hypothesis and Experiments

To test the hypothesis that requirement for significantly more drug for otherwise identical viruses targeted to R5 versus X4 expressing T-lymphocytes is related to bioavailability of intracellular PI, three specific aims (1a, 1b, 1c) were conducted, which were delineated into seven distinct questions. Specific aim 1a sought to determine the optimal conditions at which to observe peak intracellular PI levels and efflux kinetics in CD4+ T lymphocytes. This specific aim was addressed with the following questions:

- Published data demonstrates that peak intracellular PI levels in PBMC are conferred by 18 hours extracellular PI treatment at 37^{o}C. Can we recapitulate similar values in our laboratory?
- What is the inter-assay and intra-assay variability for this study?
- What are the optimal conditions to observe baseline intracellular PI levels above limit of detection in PBMC? (Variables include serum concentration in extracellular media and stimulation state of cells)
- What are the optimal conditions to observe efflux kinetics of PI in PBMC? (Variables include serum concentration in extracellular media, temperature, time, and stimulation state of cells)

Specific aim 1b sought to determine if there are enough of the R5 HIV-1 target cells within PBMC, and to determine the receptor expression profile of these cells. To address this specific aim, the following questions were posed:

- What is the receptor expression profile for subsets of T-lymphocytes for conditions that are optimal to observe intracellular PI levels in PBMC?
- The limiting factor is the R5 subset of HIV-1 target cells within the lymphocyte population. Will there be enough of this lymphocyte subpopulation under the optimized conditions?

Specific aim 1c was employed as an alternative approach to sorting cells, direct stain for efflux transporter expression, or other potential experiments discussed in the introduction of this thesis. A specific question was posed as a means to determine an alternative approach, ultimately culminating in the use of Rh-123 collaborative study, as follows:

- If the cell number is too low to obtain enough of the R5 subset, what alternative approaches can be employed to test the hypothesis?

Question 1: Confirmation of Ability to Observe Peak Intracellular PI Levels in PBMC

Intracellular bioavailability of drug is frequently determined by High Performance Liquid Chromatography (HPLC) with tandem mass spectrometric analysis to confirm the LC readout [1, 30]. Previous literature has established that peak intracellular PI levels are conferred in total PBMC upon treatment with PI containing media for 18 hours at 37°C. [1, 20,40,41,43] Before further efflux studies were performed via these methods, recapitulation of analogous experiments in the literature was performed to confirm ability to observe similar results, and to confirm that 18 hours of PI treatment yields peak intracellular PI levels.

Total PBMC were treated with 1, 10, or 100 μM IDV or RTV after 72 hr. PHA stimulation in the absence of drug, and samples were taken at 4, 18, and 48 hours post addition of drug. For PBMC treated with 1 μM IDV, intracellular IDV levels were ~3.0 μM at 4 hours and 18 hours, and decreased to ~ 2.0 μM at the end of the timecourse. For PBMC treated with 10 μM IDV, intracellular IDV levels were 13 μM at 4 hours and remained constant for the remainder of the timecourse. For PBMC treated with 100 μM IDV, intracellular IDV levels were 106 μM at 4 hours, increased to 126 μM at 18 hours, and remained constant through the remainder of the timecourse (Figure 3-1).

For PBMC treated with 1 μM RTV, intracellular RTV levels were 2.3 μM at 4 hours, increased to 5 μM at 18 hours, and decreased to 1.1 μM at the end of the timecourse. For PBMC

treated with 10 μM RTV, intracellular RTV levels were ~ 20 μM at 4 hours and 18 hours, and decreased to 12 μM by the end of the timecourse. For PBMC treated with 100 μM RTV, intracellular RTV levels were 839 μM at 4 hours, increased to $2.1x10^4$ μM at 18 hours, and decreased to ~ 900 μM by the end of the timecourse (Figure 3-1). It should be noted that technical replicates could not be performed, as the size of the study dictated use of an entire leukopack, and that this pilot study was conducted in one donor. Kinetics for IDV at all concentrations, and for RTV at 1 and 10 μM follow that of reported findings, however the kinetics for PBMC did not (Figure 3-1).

Five out of six parameters tested were analogous to published findings relative to timeframe at which peak intracellular PI levels conferred (~18 hours), and the actual intracellular μM values observed. It is hypothesized that the kinetics reported in this pilot experiment for 100 μM RTV treatment is not correct and may have encountered error. Examples of error include pipetting error, misreading of LC readout by the operator of the machinery, loading the incorrect sample into the LC for readout, or insufficient resuspension of sample before readout.

The pilot experiment conducted to answer question 1 posed in this thesis, in tandem with published literature regarding toxicity of PI in PBMC determined that 18 hours of extracellular PI treatment at 1, 10, or 100 μM PI confers peak intracellular PI levels. Specifically, intracellular PI levels are lower at timepoints observed before and after the 18-hour timepoint. Timepoint of 18 hours to confer peak intracellular PI levels in PBMC is also in concert with published literature [1, 20,40,41,43]. Specific points regarding toxicity and relationship between data obtained in this thesis to published findings and toxicity are reported in the discussion section of this thesis.

Relative to "optimal conditions" to be used for subsequent efflux studies, parameters are defined by the following parameters:

- Ability to observe intracellular PI levels above the limit of detection (which for efflux studies is ~ 1 μM)
- Time of extracellular PI treatment that confers peak intracellular PI levels
- Concentration of extracellular PI that is not toxic to the cells

This pilot study confirmed that intracellular PI levels for IDV and RTV in PBMC can be observed above the limit of detection for 1, 10, and 100 μM IDV or RTV treatment, and that the peak intracellular PI levels are conferred upon 18 hours extracellular PI treatment. Toxicity studies in the literature demonstrate that this range of concentrations (1-100 μM IDV or RTV) is not toxic to the cells and does not induce apoptosis. **The conclusion from question 1** is that all future studies with PI and PBMC will involve extracellular PI treatment within the concentration ranges 1-100 μM. This experiment should be repeated in multiple independent donors to confirm statistical significance and ability to recapitulate published findings.

Question 2: Assessing Intra and Inter-assay Variability

Upon confirmation that the employed technique yields similar results to published findings for intracellular PI levels over time, and that 18 hours of PI treatment yields peak intracellular PI levels, the next step was to perform a study to determine intra and inter-assay variability. This was achieved using U937 promonocytic cell line.

U937 cells were treated with 1 or μM RTV for 18 hours at 37^{o}C, and intracellular PI levels were quantified using the methods described above. Technical duplicates or triplicates were performed within each experiment, and the experiment was conducted in duplicate. The baseline intracellular PI levels (μM values immediately after 18 hours of extracellular 1μM RTV treatment at 37^{o}C) are reported as follows: Mean μM value within one experiment (intra-assay variability) was 3.2 μM (SEM 0.67), and the mean across experiments (inter-assay variability)

was 4.0 μM (Standard Deviation 1.5). **The conclusion from question 2** is that both intra and inter-assay variability are low (Figure 3-2).

Question 3: Determining Optimal Conditions to Observe Baseline Intracellular PI Levels Above Limit of Detection in PBMC

Question 1 was designed to test ability to recapitulate published findings, but also determined that with 1 μM extracellular PI treatment for 18 hours at 37^{o}C in 10 % serum with stimulated PBMC, intracellular PI levels are above the limit of detection. As question 1 represents a pilot experiment, and was conducted in a single donor, question 3 tested two donors and assessed two different criteria that can impact intracellular PI levels. Conducting the experiment across multiple donors and with different variables that may affect intracellular PI levels assures that optimal parameters will be determined, and that they will allow for intracellular levels above limit of detection, independent of donor tested.

PBMC were cultured with either 2 % or 10 % serum containing media, and with or without 72 hour PHA stimulation. 1 μM RTV was applied to cultures for 18 hours at 37^{o}C either immediately after PBMC isolation (for non-PHA stimulated PBMC), or after 72-hour PHA stimulation (for PHA stimulated PBMC). Baseline intracellular PI levels (immediately after 18 hour load of cells with PI) for stimulated PBMC in 2 % serum and 10 % serum were 4.7 μM (SEM 0.2) and 7.6 μM (SEM 1.6), respectively (Figure 3-3 B).

Baseline intracellular PI levels (immediately after 18 hour load of cells with PI) for non-PHA-stimulated PBMC in 2 % serum and 10 % serum were 6.6 μM (SEM 1.9) and 4.9 μM (SEM 0.2), respectively (Figure 3-3 A). **The conclusion from question 3** is that stimulation state of cells and concentration of serum in extracellular media has little effect on baseline intracellular PI levels. Experiment should be repeated in a minimum of three independent

donors, with replicates per donor, to confirm statistical significance and ability to observe results across donors.

Question 4: Optimal Conditions to Observe Efflux Kinetics of Protease Inhibitors in PBMC

The next step was to determine the optimal conditions at which to observe efflux of drug. Multiple parameters can affect efflux of drug from cells including temperature, stimulation state of cells, and time. [1,5,6,18,19,20,30,42-44]

Upon establishment that peak intracellular PI levels can be observed for stimulated and unstimulated PBMC, with 2 % or 10 % serum in extracellular media treated with RTV, the next experiment was designed to determine how serum concentration, PHA stimulation, and temperature of efflux affect efflux kinetics of PI in total PBMC. Temperature affects efflux, as ATP-dependent efflux machinery is active at 37^{o}C, and inactive at 4^{o}C. Stimulation of cells has been linked to increased efflux transporter expression, resulting in increased ability to efflux intracellular drug. Time affects efflux, as exposure of cells to inhibitory efflux conditions (4^{o}C) will initially result in inhibition of efflux (via inhibition of ATP-dependent efflux machinery). Cells loaded with drug will, as a function of time, begin to passively diffuse drug over time, independent of temperature. [1,5,6,18,19,20,29,42-44]

Optimizing these conditions would allow for determination of which combination of serum, stimulation, and efflux temperature would best facilitate observing differences in baseline as well as efflux kinetics in subsets of PBMC. Stimulated PBMC were treated with media containing 1 or 10 uM RTV and 10 % serum for 18 hrs. After 18 hr. RTV treatment, efflux was observed at 4^{o}C or 37^{o}C at baseline (immediately after 18 hour RTV load at 37^{o}C), 10, 20, 40, and 60 minutes. Data for 10 μM RTV is n=1, and 1 μM RTV is n=2 with technical duplicates, however only one sample was sent to Denver, CO., for analysis. Independent of extracellular

concentration of RTV applied, the majority of drug was retained intracellularly throughout the timecourse at 4°C (Figure 3-4, blue circles, red circles), while nearly all PI effluxed to levels below limit of quantitation (Below Limit of Quantitation [BLQ]; ~ 1 uM) within the first ten minutes at 37°C (Figure 3-4, blue triangles, red triangles). These findings demonstrated that RTV was able to enter cells, as baseline values were 7.6 μM (+/-1.6 μM) and 12.2 μM (+/-3.4 μM) for 1 or 10 uM RTV treatment respectively (Figure 3-5), but that efflux occurs very rapidly as a function of 37°C temperature (Figure 3-4, blue triangles, red triangles).

Treatment of stimulated PBMC with 1 uM RTV in 10 % serum did not yield conditions at which efflux can be observed above limit of detection, therefore parameters were modified. A study was then performed with a reduced serum concentration (2 %) and performed the efflux at an intermediate temperature (20°C) as well as 4°C and 37°C, using stimulated and unstimulated PBMC treated with 1 uM RTV for 18 hrs. As a control, condition of 10 % serum was tested again. Data for 2 % serum is n=1.

All data including baseline intracellular levels were BLQ. Hypothesized reasons for error and discussion regarding these possible errors have been removed from this thesis. For experiment with 2 % serum, as expected, nearly all PI was maintained intracellularly throughout the timecourse independent of stimulation criteria for cells effluxed at 4°C (Figure 3-6 A, B, short, green lines) with the exception of the 120 minute timepoint for stimulated PBMC. The 120-minute timepoint intracellular level proved to be irrelevant, as nearly all RTV was effluxed from the cells within 10 minutes at 37° C for stimulated PBMC (Figure 3-6 A, blue lines, filled circles) and unstimulated PBMC (Figure 3-6 B, blue lines, filled triangles). For cells effluxed at 20°C, ~ 50 % of RTV was effluxed within the first ten minutes and RTV had effluxed BLQ by 120 minutes for unstimulated PBMC (Figure 3-6 B, red line, filled triangle). For stimulated

PBMC effluxed at 20°C, ~ 15 % of RTV had effluxed by the 10 minute timepoint, whereas nearly all RTV had effluxed by the 20 minute timepoint and remained BLQ for the remainder of the timecourse (Figure 3-6 A, red line, filled triangles). BLQ is ~ 1 μM. Data are n=1 with technical duplicates/donor, but only one replicate was sent to Denver, CO., for analysis. No follow up experiments were performed to confirm efflux pattern of RTV in PBMC resuspended in 2 % serum in other independent donors.

The conclusion drawn from question 4 is that optimal conditions to observe baseline intracellular levels and efflux kinetics for PBMC include use of unstimulated PBMC maintained in 2 % serum and effluxed at 20°C, but that experiment should be repeated in a minimum of three independent donors, with replicates per donor, to confirm statistical significance and ability to observe results across donors.

Question 5: Receptor Expression Profile For Subsets of T-lymphocytes in Optimal Conditions to Observe Intracellular Protease Inhibitor Levels in PBMC

Question 4 determined that the optimal parameters to observe intracellular PI levels are in unstimulated PBMC in 2 % serum. A study was performed in three independent donors with technical duplicates/donor, however data from only one replicate was saved and analyzed (summary of data for all donors in Table 3-1) to determine the receptor expression profile of CD4, CXCR4 and CCR5, within a population of unstimulated lymphocytes enriched for CD4 expression and depleted of CD14 expressing cells (monocytes, which also express CD4).

In the CD4 enriched population CD4 enriched T lymphocytes were subjected to gating on CD4, demonstrating that ~ 80 % of enriched cells were CD4 positive (Figure 3-7 A).
A gate was drawn around these CD4 positive cells, and that population of cells was subjected to CD14 gate, demonstrating that zero percent of CD4 positive cells were CD14 positive (Figure 3-

7 B). This demonstrates that none of the CD4 positive cells are monocytes, which express CD14.

Next, the CD4 positive population was subjected to gate to determine percentage of CXCR4+, CCR5+, and CXCR4+/CCR5+ cells. Gate was established based on isotype matched controls. It was determined that CXCR4+/CD4+ lymphocytes represent ~ 80 % of the CD4+ lymphocyte population (Figure 3-7 C). CCR5+/CD4+ lymphocytes represent ~ 20 % of CD4+ lymphocyte population (Figure 3-7 D), and that the CCR5+ cells are dim, and do not display significant delineation between CCR5+ and CCR5- subpopulations (Figure 3-7 D). ~ 15 % of the CD4+ lymphocytes were CD4+/CXCR4+/CCR5+ (Figure 3-7 E). Data are representative from one of three independent donors tested. All three donors displayed values that are within two standard deviations of the mean. All values obtained were +/- 7 % of each other. Summary of data from the three donors tested appear in Table 3-1. Pilot study to increase intensity of CCR5 signal with FASER kit (Miltenyi Biotec, Auburn, CA), which provides an excellent foundation to determine methods to obtain distinct CCR5+ population, was not performed. Further studies to modify stimulation and media conditions of cells to increase CCR5 expression, and decrease double positive X4+/R5+ subpopulations were not performed.

Question 6: Are There Enough CCR5 Cells to Perform Efflux Study in Unstimulated PBMC?

As CCR5 expression is low in unstimulated PBMC, it was mathematically calculated to be possible, but decided to be unfeasible, to obtain the necessary number of cells from one leukopack of freshly isolated, non PHA-stimulated PBMC and examine efflux differences between R5 and X4 cells. In addition, CCR5 positive lymphocytes display dim receptor expression, which presents significant difficulty in sorting this population of cells, and differentiating this population of cells from isotype, or from CXCR4+ cells, impeding ability to

set gate around population of CCR5+ cells with confidence. Pilot study to increase intensity of CCR5 signal with FASER kit (Miltenyi Biotec, Auburn, CA), which provides an excellent foundation to determine methods to obtain distinct CCR5+ population, was not performed. Further studies to modify stimulation and media conditions of cells to increase CCR5 expression, and decrease double positive X4+/R5+ subpopulations were not performed.

One leukopack can yield ~ $7.0x10^8$ PBMC, of which ~ 50 %, or ~ $5.6x10^8$ are CD4+ lymphocytes, and of which ~$7.0x10^7$ cells are CD4+/CCR5+/CXCR4-. For one efflux study, ~$5.0x10^6$ cells/condition is a minimum requirement. Twelve conditions are required for efflux study (baseline, 10, 20, 40, 60, and 120 minute timepoints) as well as counter cells for 4^oC and 37^oC (tubes which are treated analogously to all other cells, but whose sole purpose is to obtain an accurate cell count at the conclusion of the study). Thus, 14 parameters are necessary, totaling ~ 7.0 x 10^7 CD4+/CCR5+/CXCR4- lymphocytes. In order to obtain these target cells, a live gate cell sort must be conducted. These calculations represent a conservative calculation, and experiments to study smaller total number of cells for efflux study, and to determine if $7.0x10^7$ could be feasible for efflux studies were not conducted. Antibodies for CD4, CCR5, CXCR4, and CD14 must be used. To stain the ~ 5.6 x 10^8 CD4+ lymphocytes isolated with each of these markers would total ~ $2,000 ($260/antibody X 6.5 total bottles of Ab (1.3 bottles of anti-CD4+ 1.3 bottles of anti-CXCR4 + 1.3 bottles anti-CCR5 + 1.3 bottles of anti-CD14 + 1.3 bottles-isotype matched controls) + ($50/hr flow cytometer X 3.5 hours at flow rate demonstrated to not induce apoptosis in lymphocytes). Viability of sorted cells was not tested, and the expectation is that not 100 % if cells would be viable after undergoing sort. The number of calculated R5 target cells not accounting for loss of unviable cells after the sort is ~20-40 million cells more than the threshold requirement for the efflux study. Although the method of

sorting coupled with subsequent efflux study could have mathematically provided enough cells to perform the efflux study, this idea was terminated in favor of alternative method (Rh-123 assay) described below.

Further studies to obtain a flurochrome which does not significantly overlap with isotype, presenting brighter CCR5 receptor expression profile, coupled with additional studies including viability assessment over time of sorted cells and repetition of study in multiple independent donors were not conducted. Studies including modification of stimulation criteria for cells and media conditions that may decrease double positive X4+/R5+ lymphocytes were not performed, but present an excellent foundation from which to launch further studies to obtain distinct populations of X4 and R5 HIV-1 target cells within the lymphocyte population. Due to these limitations/stringencies, an alternative approach was employed to observe p-glycoprotein activity within populations of cells representing the X4 and R5 HIV-1 target cells within the lymphocyte population via flow cytometry.

Question 7: Alternative Approach and Rh-123 Assay

Many alternative methods and approaches could have been employed as an alternative method, as discussed in the introduction, and addressed in the discussion. The Rh-123 study was chosen as an alternative approach to sorting subpopulations of HIV-1 target cells within the lymphocyte population. As part of a collaborative study, the experiments in this section were performed by Matt Morrow in the laboratory of Dr. John Sleasman, University of South Florida. Rh-123 can be fluorescently detected via FL1 and is a substrate for p-gp[68]. Therefore, cells that are p-gp negative will retain Rh-123 (Rh-123 bright) whereas cells that express p-gp will efflux Rh-123 (Rh-123 dim). Rh-123 provides a mechanism to observe p-gp activity and was used to assess p-gp activity in subsets of HIV-1 target cells within the lymphocyte population. Rh12-3

in tandem with PI treatment to cells was then employed to confirm that PI are substrates for p-gp, and that this could be observed in the eight-color flow analysis that was used in this study.

P-gp Activity in Lymphocyte Subsets

To explore p-gp activity as a potential mechanism for differences in PI levels required to inhibit replication in R5 versus X4 lymphocytes, Rh-123 efflux was assessed in several subsets of lymphocytes. Initial gates were established to determine Rh-123 fluorescence in total PBMC and demonstrated heterogeneous p-gp activity within the lymphocyte population (Figure 3-8). Further gating on subpopulations of lymphocytes was then established. CD4+ lymphocytes, NK cells (CD16+/CD56+), and CD8+ T cells. Total PBMC, CD4+ T- lymphocytes, and CD8+ T lymphocytes contain cells with heterogeneous p-gp expression, and NK cells display high p-gp expression (highest of any known human cell) (Figure 3-9.1 A, B, C). Separate gates were established on total PBMC to obtain $CD3^{+}/CCR5^{+}$ and $CD3^{+}/CCR5^{-}$ cell populations (Figure 3-9.2 D). Further gating was established to obtain CD45RA+/- and CD45RO+/- lymphocytes within the CD3/CCR5 positive and negative populations (Figure 3-9.2 E, F).

Each of these subsets represents either the X4 or R5 HIV-1 target cells within the lymphocyte population. A subset of cells representing the R5 HIV-1 target cells demonstrated the lowest Rh-123 fluorescence relative to all other subsets tested (Figure 3-9.2 F, circled population). As Rh-123 fluorescence is inversely proportional to p-gp activity, this result indicated high p-gp expression analogous to NK cell p-gp expression in this population of cells relative to all other subsets of lymphocytes tested. This experiment was conducted in one donor without technical replicates.

Protease Inhibitors are Substrates for p-gp

The next goal was to demonstrate that IDV and RTV are substrates for p-gp within the confines of our experimental conditions, thus establishing that p-gp levels correlate with

intracellular PI levels. Although published literature defines this fact, no study has addressed this question with eight-color analysis. Therefore, confirmation of the ability to recapitulate this observation with these additional parameters was performed.

Rh-123 activity was assessed in the presence of IDV, RTV, and known inhibitor of p-gp, R-Verapamil. Molecules with higher affinity for p-gp than Rh-123 will displace Rh-123 and thus preferentially efflux from cells. Therefore, p-gp positive cells in the presence of Rh-123 and substrate of higher affinity than Rh-123 will be Rh-123 bright. CD3+/CD4+/CCR5+ lymphocytes subsequently gated on CD45RA and CD45RO expression were loaded with Rh-123 for 30 minutes (Figure 3-10 A, shaded), allowed to efflux for 1 hour at 37^{o}C after Rh-123 load (Figure 3-10 A, red), allowed to efflux for 1 hour in the presence of 10 μM R-Verapamil, IDV, or RTV (Figure 3-10 A, blue). Treatment with R-Verapamil, IDV or RTV was sufficient to inhibit Rh123 efflux in all populations of cells (Figure 3-10 B). These findings demonstrated that IDV and RTV are substrates for p-gp and that this interaction can be defined with a complex panel of fluorochromes. Together, these findings further validate the hypothesis that differential p-gp activity directly correlates with variable levels of PI found within lymphocyte subsets. This experiment was conducted in one donor without technical replicates. This experiment must be repeated in multiple independent donors to confirm statistical significance and ability to observe results across donors.

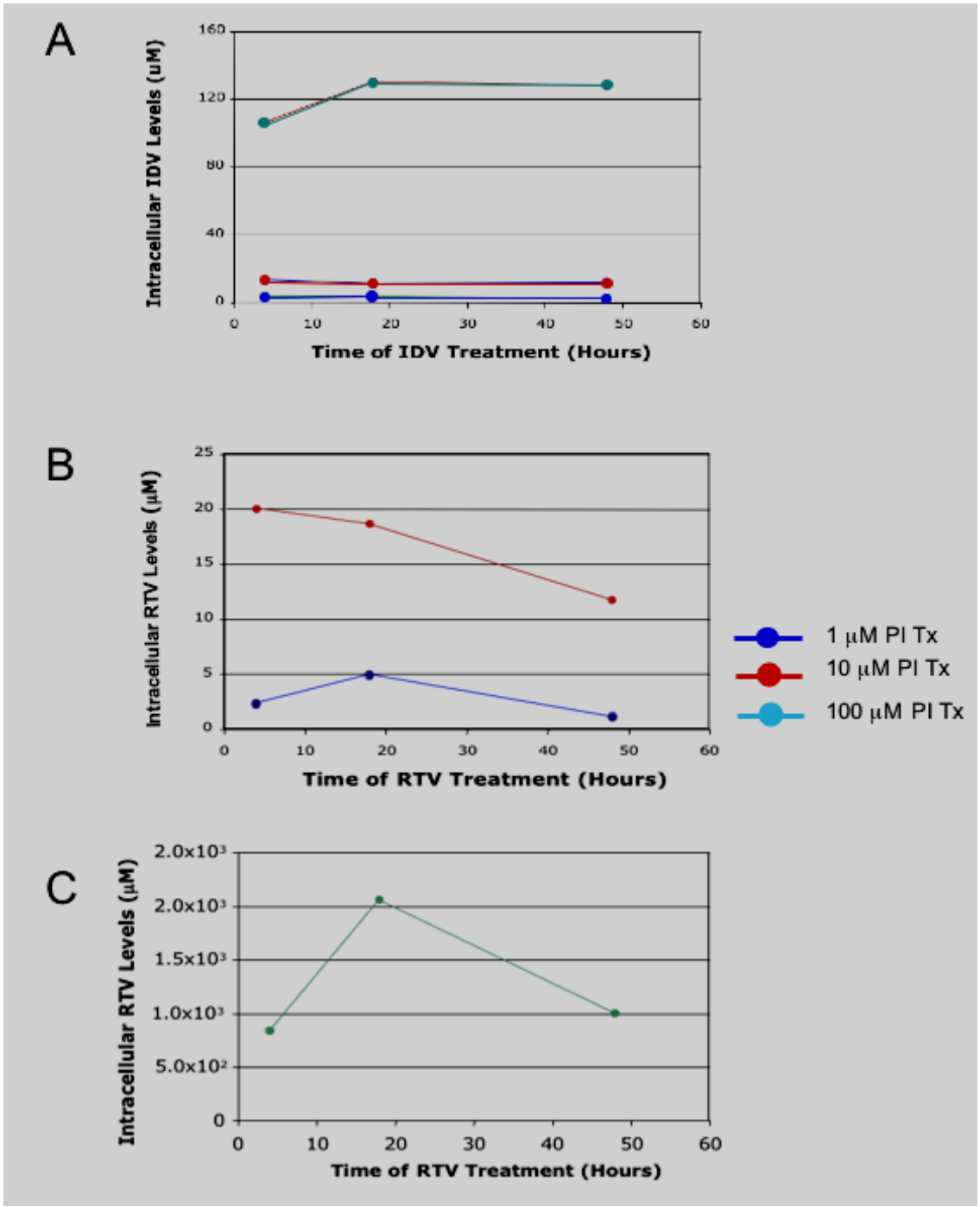


Figure 3-1. Peak intracellular PI levels in PBMC treated with 1, 10, 100 µM IDV or RTV. Peak intracellular PI levels were conferred upon ~ 18 hours extracellular PI treatment for both IDV and RTV, independent of extracellular concentration applied (A, B, C). This experiment represents n=1, without technical replicates. Blue = 1 µM PI tx, red = 10 µM PI tx, green = 100 µM PI tx.

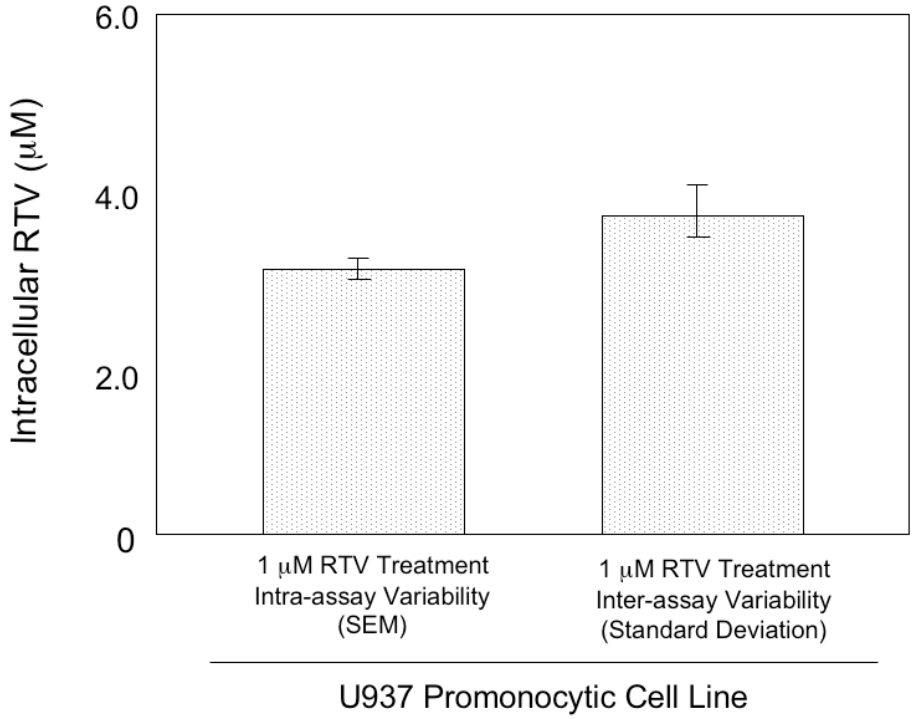


Figure 3-2. Intra-assay and inter-assay variability in PI efflux study. U937 were treated with 1 μM RTV, and baseline intracellular PI levels were observed after 18 hours extracellular PI treatment. This figure contains data from two independent experiments with technical duplicates or triplicates per experiment. Error bars for the data on the left bar indicate SEM and error bars for the data on the right indicate standard deviation. Mean μM value within one experiment (intra-assay variability; left bar) was 3.2 μM (SEM 0.67), and the mean across experiments (inter-assay variability; right bar) was 4.0 μM (Standard Deviation 1.5), and all values obtained fell within two standard deviations of the mean, demonstrating that both variability is low.

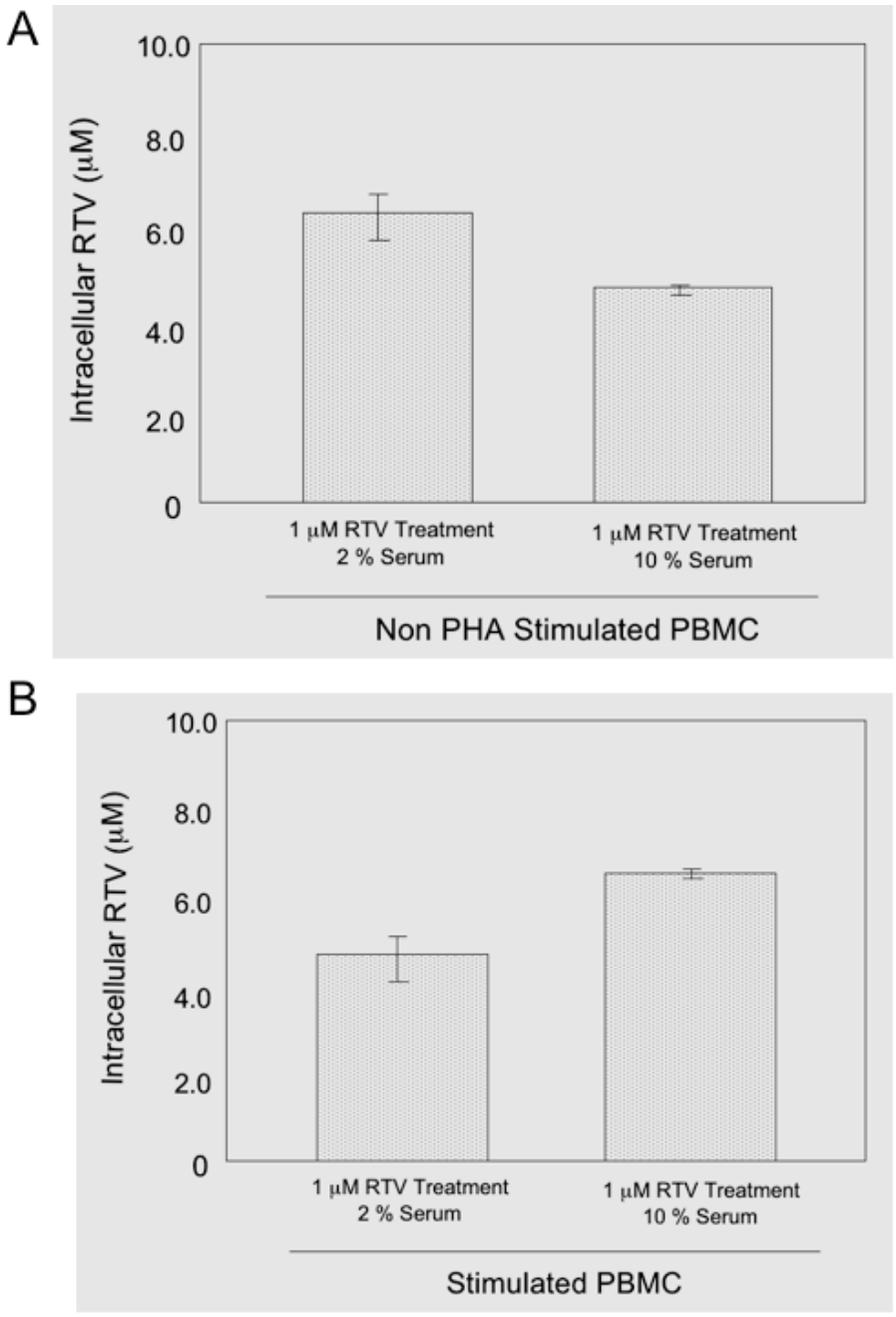


Figure 3-3. Baseline intracellular RTV levels. (A) For unstimulated, (B) for PHA stimulated PBMC treated with 1 μM RTV treatment for 18 hours at 37^{o}C in 2 % or 10% serum. Baseline intracellular PI levels (immediately after 18 hour load of cells with PI) for non-PHA-stimulated PBMC in 2 % serum and 10 % serum were 6.6 μM (SEM 1.9) and 4.9 μM (SEM 0.2), respectively (A). Baseline intracellular PI levels (immediately after 18 hour load of cells with PI) for stimulated PBMC in 2 % serum and 10 % serum were 4.7 μM (SEM 0.2) and 7.6 μM (SEM 1.6), respectively (B). Data from 10 % serum is from two donors, with technical duplicates/donor. Data from 2 % serum is from one donor, with technical duplicates/donor. Error for data indicates SEM.

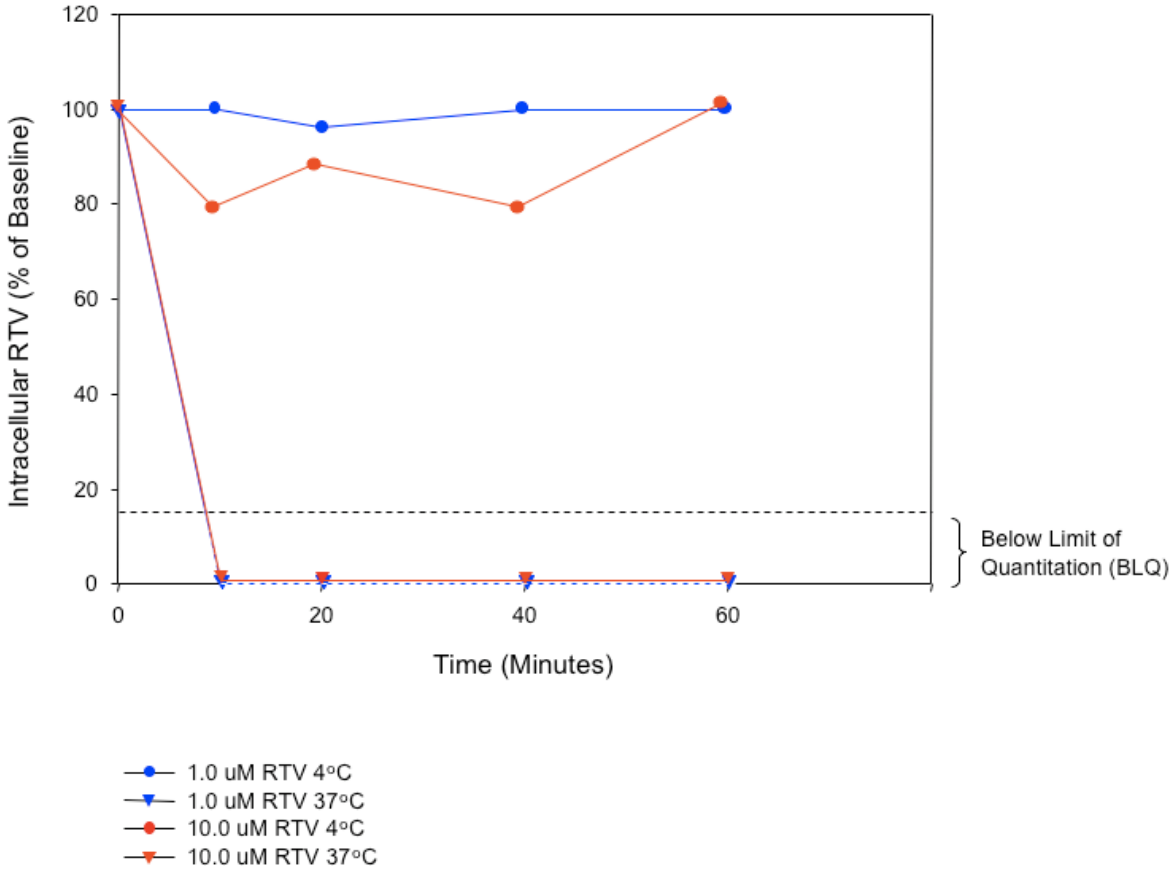


Figure 3-4. Efflux of RTV in stimulated PBMC. Nearly all RTV remained intracellular throughout the timecourse for cells maintained at 4°C (filled circles), whereas nearly all RTV was effluxed from the cells within 10 minutes at 37°C (filled triangles), independent of extracellular concentration of RTV applied. Red lines indicate 10 µM extracellular RTV treatment whereas blue lines indicate 1 µM RTV extracellular treatment. Data for 1.0 µM RTV is representative from one donor of two donors tested. Data from the second donor treated with 1.0 µM were all BLQ. Data for 10.0 µM RTV is n=1. Technical duplicates/donor were conducted, but only one replicate was sent to Denver, CO., for analysis. BLQ is ~ 1 µM.

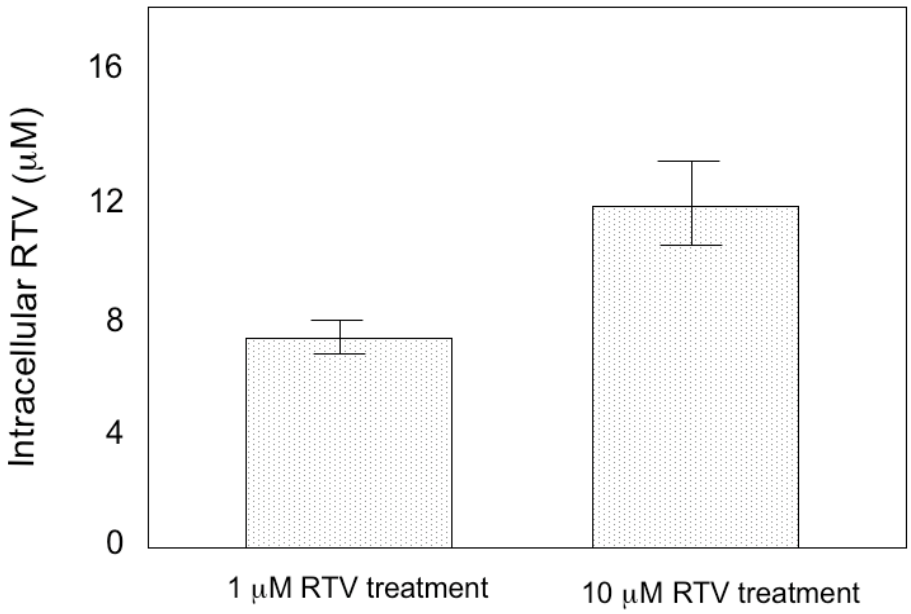


Figure 3-5. Baseline intracellular RTV levels from Protease Inhibitor efflux study. Although nearly all RTV had effluxed from cells within 10 minutes at 37°C, baseline intracellular RTV levels were significantly above the limit of detection. For 1μM RTV treatment, intracellular RTV levels were nearly 8 μM, and for 10 μM RTV treatment, intracellular levels were ~ 12 μM (left bar, right bar, respectively). Data are from two independent donors, with technical duplicates/donor. Error represents SEM.

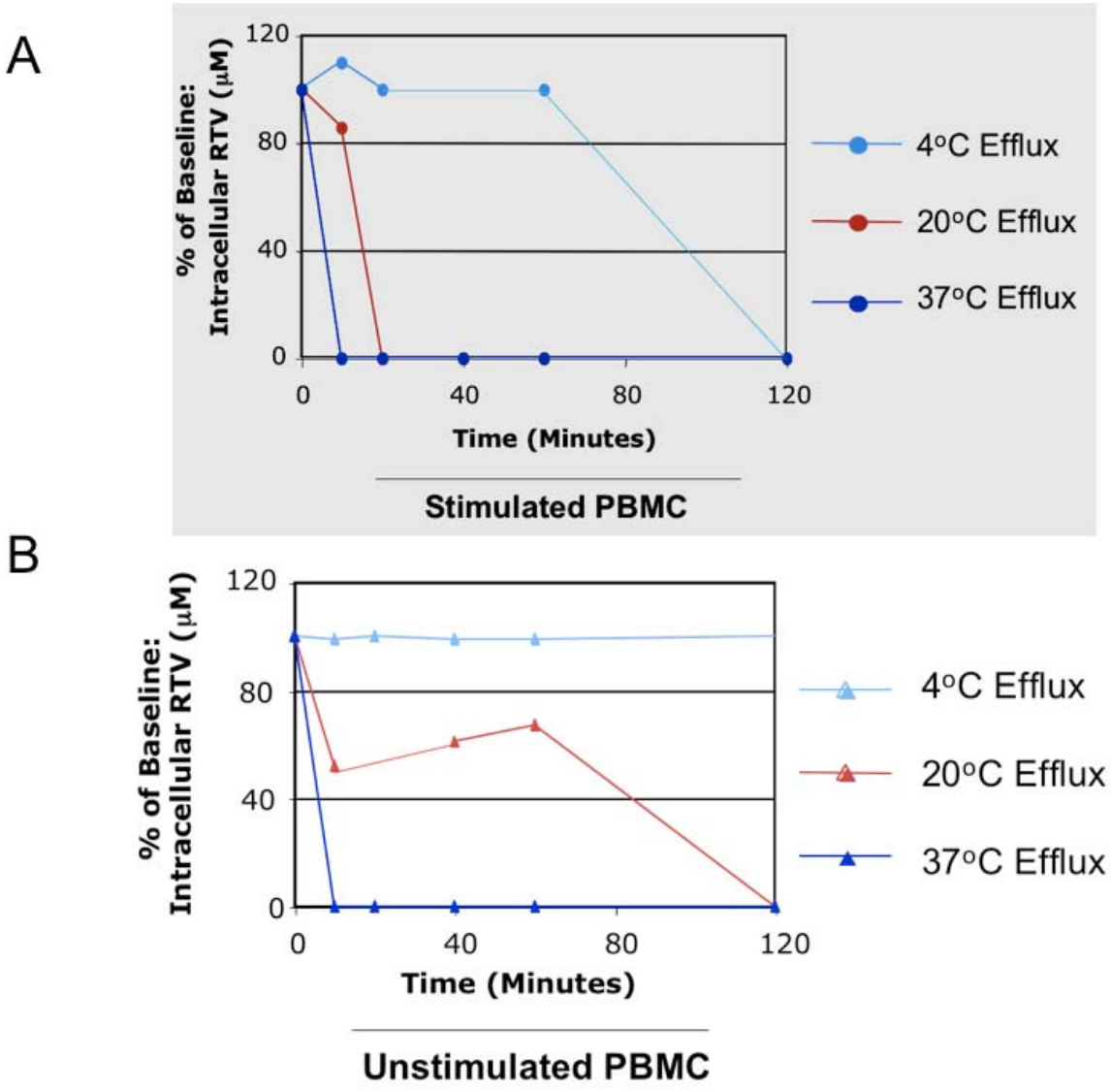


Figure 3-6. Efflux of RTV in stimulated or unstimulated PBMC in 2 % serum. Nearly all RTV remained intracellular throughout the timecourse for cells maintained at 4°C for stimulated PBMC (A, green lines, filled circles) and unstimulated PBMC (B, green lines, filled triangles) with the exception of the 120 minute timepoint for stimulated PBMC which was BLQ. Nearly all RTV was effluxed from the cells within 10 minutes at 37° C for stimulated PBMC (A, blue lines, filled circles) and unstimulated PBMC (B, blue lines, filled triangles). For efflux at 20°C, ~ 50 % of RTV was effluxed within the first ten minutes and RTV had effluxed BLQ by 120 minutes for unstimulated PBMC (B, red line, filled triangle). For stimulated PBMC effluxed at 20°C, ~ 15 % of RTV had effluxed by the 10 minute timepoint. Nearly all RTV had effluxed by the 20 minute timepoint and remained BLQ for remainder of the timecourse (A, red line, filled triangles). BLQ is ~ 1 μM. Data are n=1 with technical duplicates/donor, but only one replicate was sent to Denver, CO., for analysis.

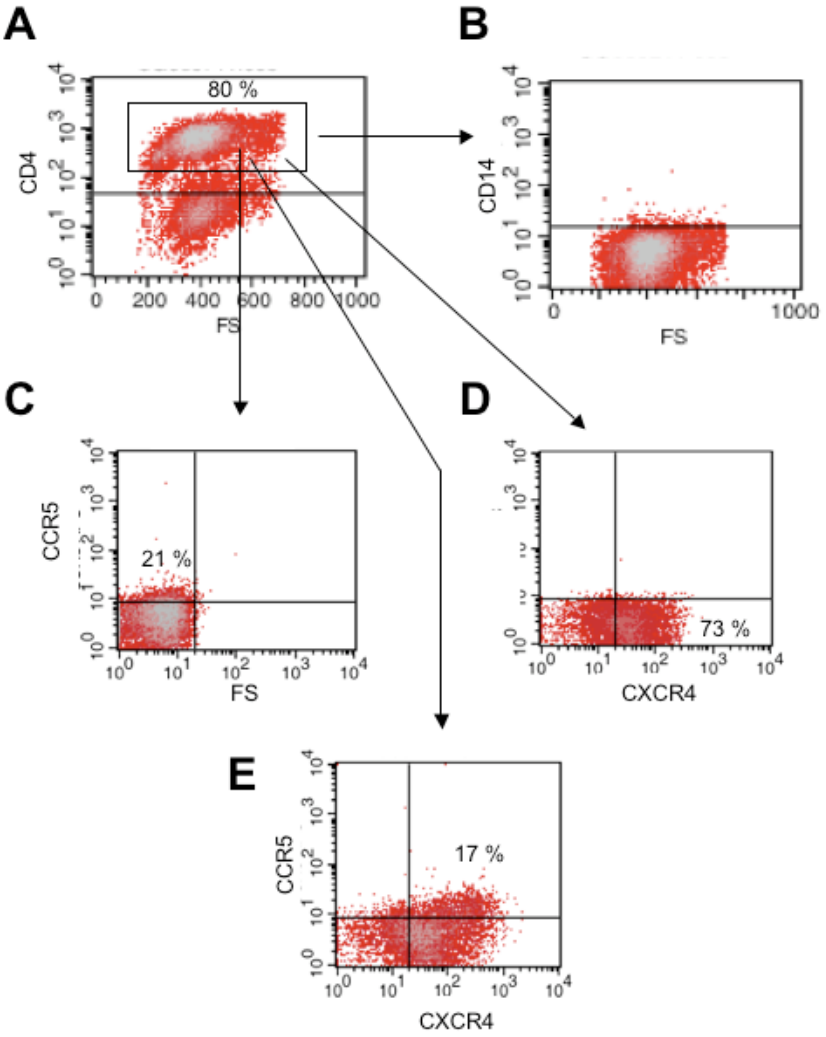


Figure 3-7: Receptor expression profile for CD4 enriched lymphocytes. CD4 enriched population was gated on CD4 expression (A). ~ 80 % of enriched cells were CD4 positive. CD4 positive lymphocytes were then subjected to gate on CD14 (B). None of the CD4 enriched cells expressed CD14 (monocytes) (B). CD4 positive lymphocytes were subjected to gate on CXCR4 (C), CCR5 (D), or dual plot for CXCR4/CCR5 (E). Percent of CD4/CXCR4 cells is ~ 75 % of enriched population, and percent of CD4/CCR5 cells is ~ 20 % of enriched population. Percent cells positive for both CXCR4 and CCR5 is ~15 % (E). All gates were established for isotype matched controls. Data are representative from one of three independent donors tested. All three donors displayed values that are within two standard deviations of the mean, indicating statistically insignificant difference in values obtained. Summary of data from the three donors tested appear in Table 3-1.

Table 3-1: Receptor expression profile for CD4 enriched lymphocytes: Summary from three independent donors. Percent of CD4 enriched cells that are CD4+, CD4+/CXCR4+, CD4+/CCR5+, and CD4+/CCR5+/CXCR4+. All three donors displayed values that are within two standard deviations of the mean, indicating statistically insignificant differences in values obtained.

	Donor 1 ***(CD4+ enriched lymphocytes)***	***Donor 2*** ***(CD4+ enriched lymphocytes)***	***Donor 3*** ***(CD4+ enriched lymphocytes)***
% CD4+ Cells	80	83	81
% CD14+ Cells	0	0	0
% CD4+/CXCR4+ Cells	**73**	**83**	**72**
% CD4+/CCR5+ Cells	**21**	**24**	**28**
% CD4+/CXCR4+/CCR5+ Cells	**17**	**20**	**17**

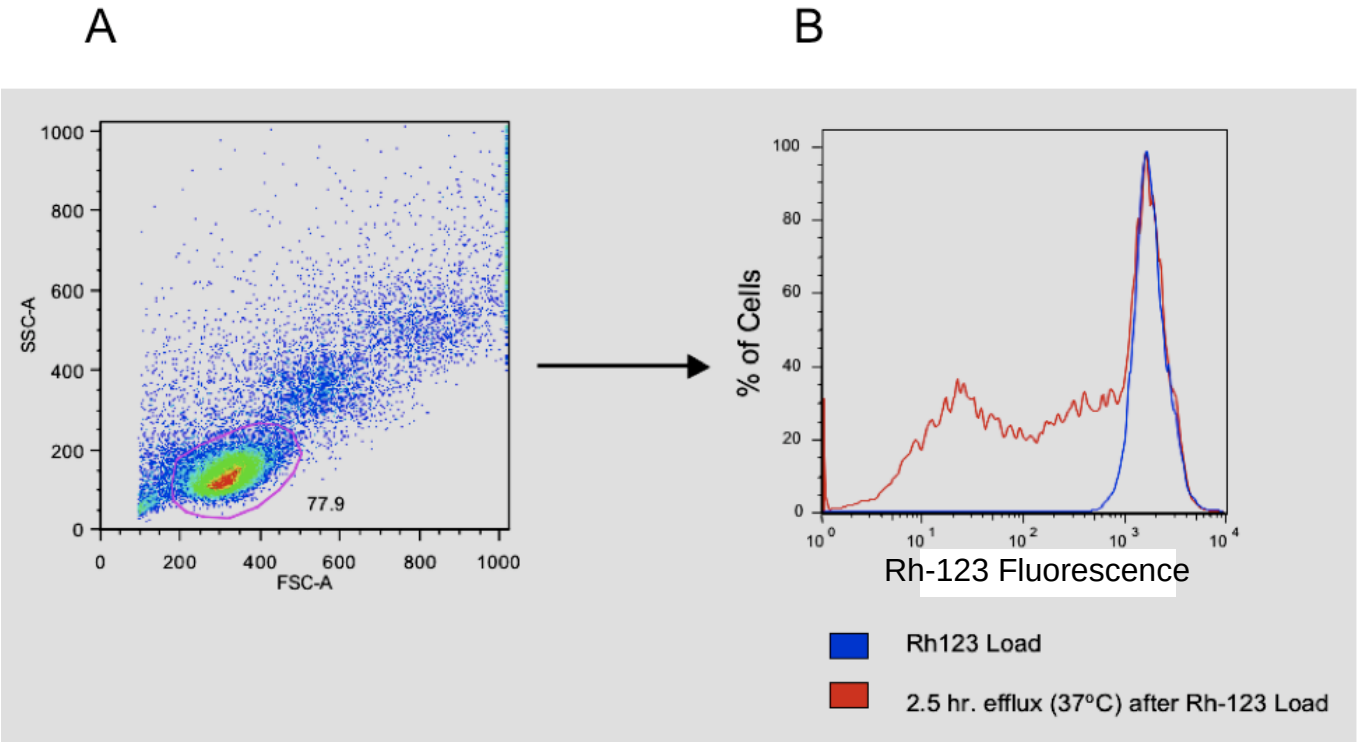


Figure 3-8. Establishment of gate and Rh-123 efflux in total PBMC. Gate was drawn around lymphocyte population (A). Rh-123 loaded cells displayed high Rh-123 fluorescence (B, blue), whereas lymphocytes displayed heterogeneous Rh-123 fluorescence (B, red). Data represent one donor tested without technical replicates.

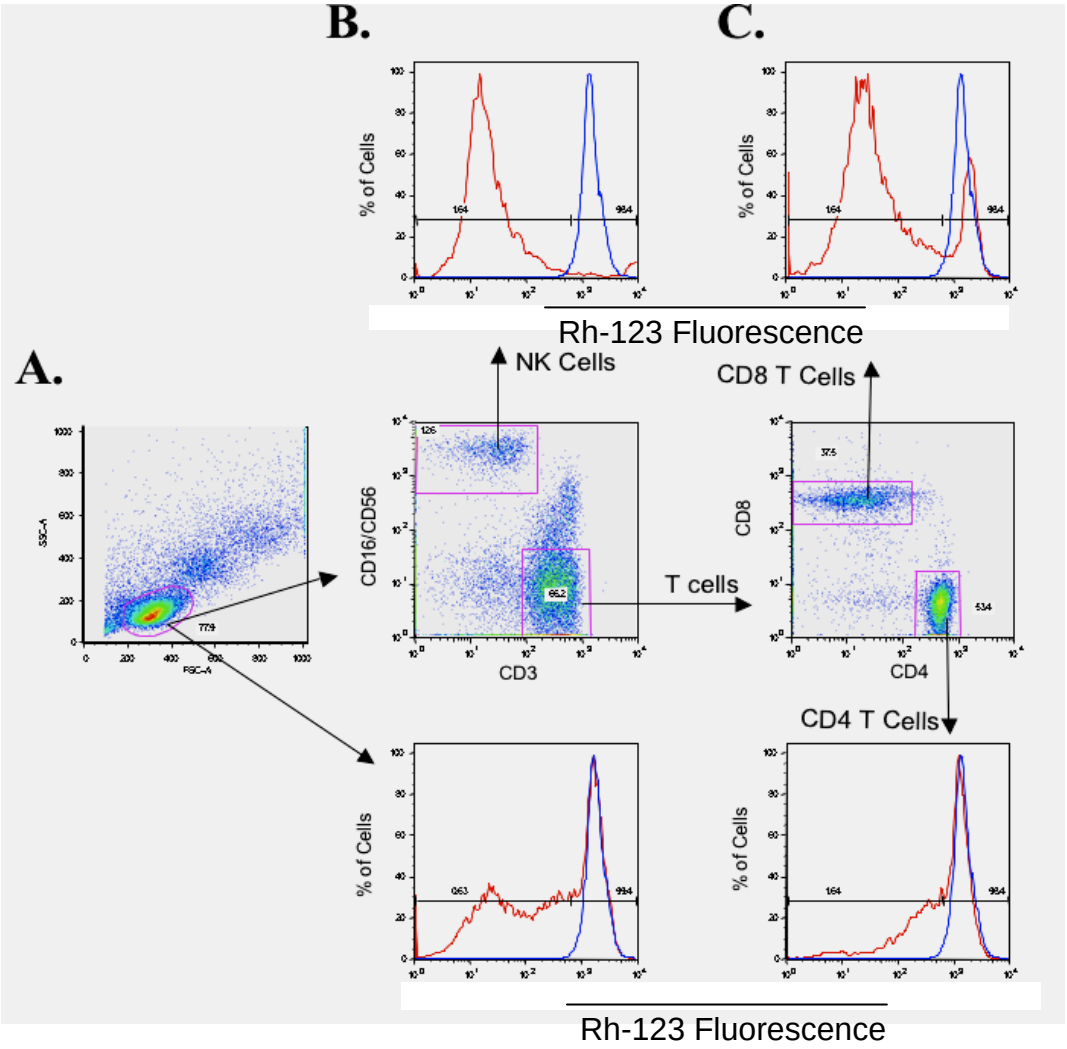


Figure 3-9.1. Rh-123 activity in NK cells, CD4 and CD8 T lymphocytes. Gates were established to determine Rh-123 fluorescence in NK cells, CD8, CD4, cells. NK cells display low Rh-123 fluorescence. CD8 cells display heterogeneous Rh-123 fluorescence, and exhibit two distinct populations; one with high Rh-123 fluorescence one with lower Rh-123 fluorescence. CD4 cells display heterogeneous Rh-123 fluorescence and appear to contain a mixture of cells; some with high Rh-123 fluorescence (overlap with Rh-123 loaded cells), some with lower Rh-123 fluorescence (overlap with NK cells or CD8 cells). Data represent one donor tested without technical replicates.

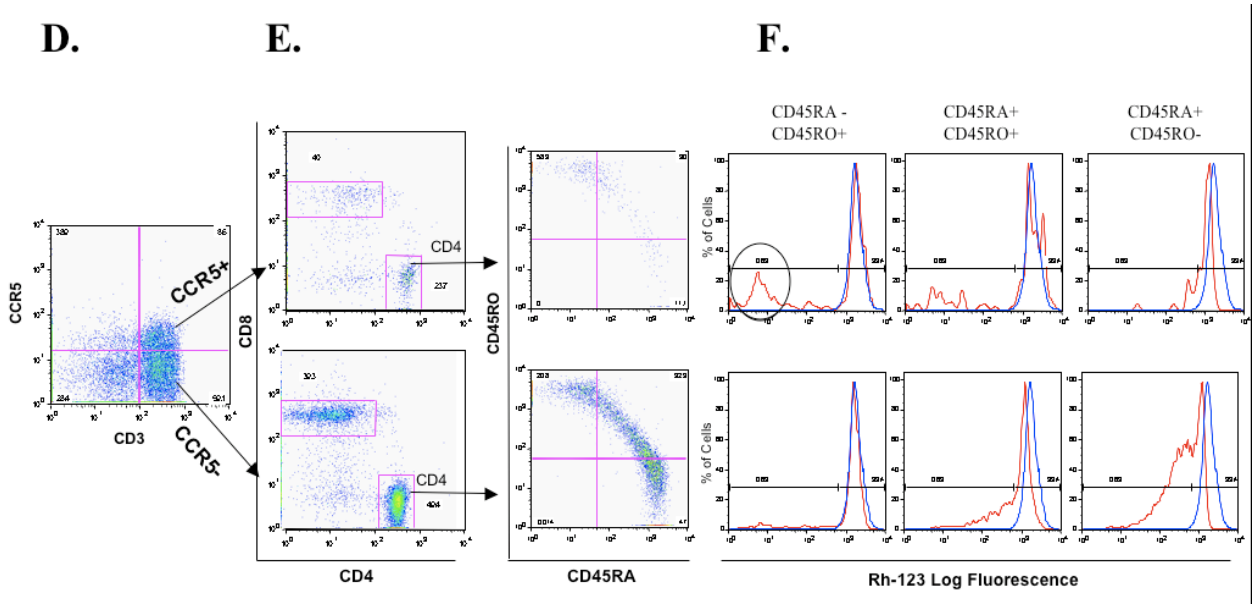
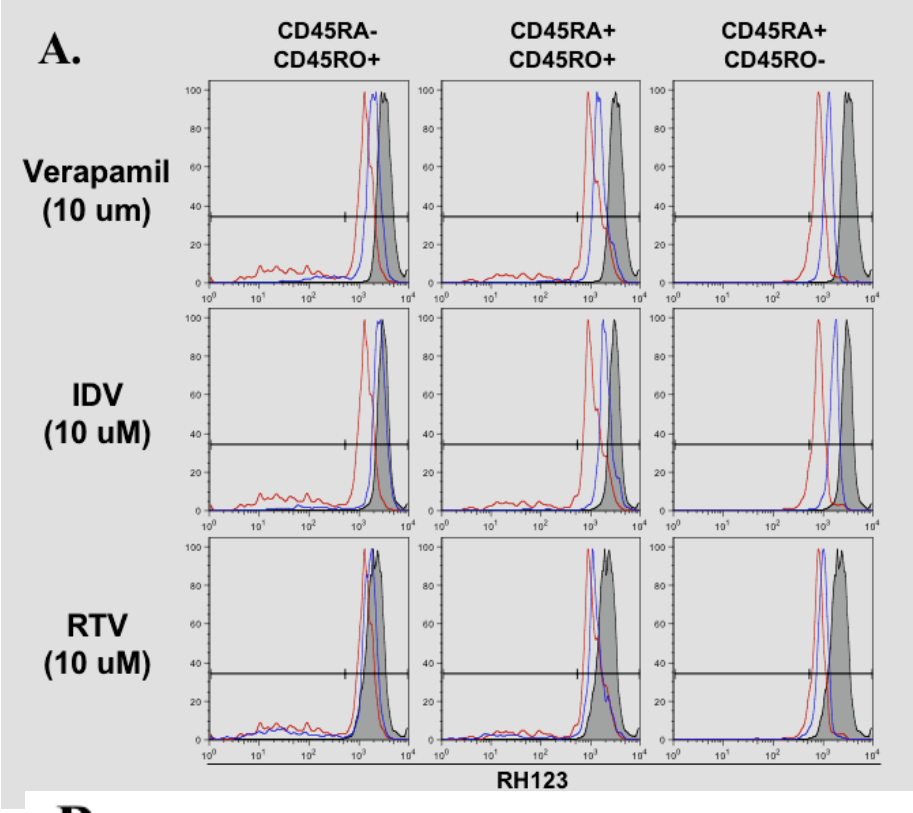


Figure 3-9.2. Rh-123 activity in subsets of HIV-1 target cells. Separate gates were established on total PBMC to obtain $CD3^{+}/CCR5^{+}$ and $CD3^{+}/CCR5^{-}$ cell populations (D). Further gating was established to obtain CD45RA+/- and CD45RO+/- lymphocytes within the CD3/CCR5 positive and negative populations (E, F). A subset of cells representing the R5 HIV-1 target cells demonstrated the lowest Rh-123 fluorescence relative to all other subsets tested (F, circled population. Data represent one donor tested without technical replicates.



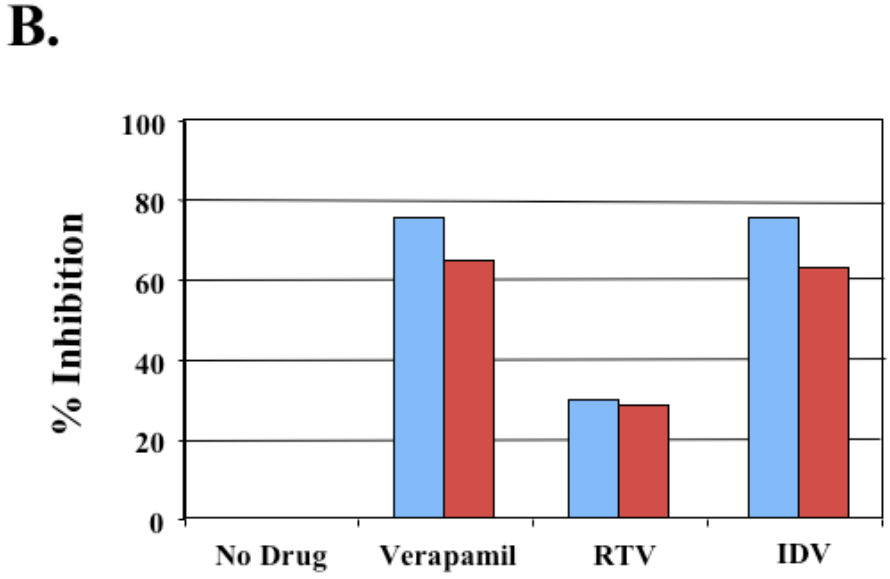


Figure 3-10. Efflux of Rh-123 in the presence of IDV, RTV, or R-Verapamil. CD3+/CD4+/CCR5+ lymphocytes subsequently gated on CD45RA and CD45RO expression were loaded with Rh-123 for 30 minutes (A, shaded), allowed to efflux for 1 hour at 37°C after Rh-123 load (A, red), allowed to efflux for 1 hour in the presence of 10 µM R-Verapamil, IDV, or RTV (A, blue). Efflux was observed at one hour (B, red), or two hours (B, blue). Treatment with R-Verapamil or IDV inhibited ~80 % of Rh-123 efflux. RTV inhibited ~ 30 % of Rh-123 efflux (B). Data represent one donor tested without technical replicates.

CHAPTER 4
DISCUSSION

Questions Posed to Test Hypothesis of Thesis

The **hypothesis** of this thesis is that decreased intracellular bioavailability of PI within the R5 relative to X4 lymphocyte subsets is a possible mechanism responsible for requirement of significantly higher IC_{50} in the R5 subset. PI are substrates for multiple efflux transporters, most notably p-glycoprotein (p-gp), a member of the ABC Efflux transporter family. ABC efflux transporters are highly variable throughout tissue and between cell types. [1,2,4-6,11,18-20,22,33,37,42-44,60,64,66,68,71,73] A series of specific aims were designed, with specific questions posed to test the hypothesis as follows:

Specific Aim 1a was designed to determine optimal conditions to observe peak intracellular PI levels and efflux kinetics in CD4+ T lymphocytes, and was answered with the following questions:

- Published data demonstrates that peak intracellular PI levels in PBMC are conferred by 18 hours extracellular PI treatment at 37^{o}C. Can we recapitulate similar values in our laboratory?
- What is the inter-assay and intra-assay variability for this study?
- What are the optimal conditions to observe baseline intracellular PI levels above limit of detection in PBMC? (Variables include serum concentration in extracellular media and stimulation state of cells)
- What are the optimal conditions to observe efflux kinetics of PI in PBMC? (Variables include serum concentration in extracellular media, temperature, time, and stimulation state of cells)

Specific aim 1b was designed to determine if there are enough R5 HIV-1 target cells in the lymphocyte population to perform sorting. To answer this question, the following questions were posed:

- What is the receptor expression profile for subsets of T-lymphocytes for conditions that are optimal to observe intracellular PI levels in PBMC?

- The limiting factor is the R5 subset of HIV-1 target cells within the lymphocyte population. Will there be enough of this lymphocyte subpopulation under the optimized conditions?

Specific aim 1c was designed as an alternative approach, as other follow up experiments and methods were not performed. This aim sought to determine the p-gp activity in subsets of lymphocytes representing X4 or R5 HIV-1 targets. This aim employed a Rh-123 flow cytometric based assay, which focused upon the following question:

- If the cell number is too low to obtain enough of the R5 subset, what alternative approaches can be employed to test the hypothesis?

Relating Peak Intracellular PI Data to Toxicity and Published Data

Relative to the extracellular concentration of PI applied in this pilot study, previous studies in the literature have demonstrated that up to ~ 400 μM extracellular treatment of PBMC with PI does not result in apoptosis, as determined by multiple methods to observe both mitochondrial and downstream indicators of apoptosis (DNA fragmentation). [87, 88,91,102,113] Studies conducted in the Goodenow laboratory focusing solely on mechanism related observed with the CellTiter-Blue® Assay (Promega, Madison, WI) which is based on the ability of living cells to convert a redox dye (resazurin) into a fluorescent end product (resorufin). Viable cells retain the ability to reduce resazurin into resorufin. Nonviable cells rapidly lose metabolic capacity, do not reduce the indicator dye, and thus do not generate a fluorescent signal [3,47]. This marker for apoptosis is downstream of early indicators of apoptosis such as Annexin V, but upstream of markers for late stage apoptosis including DNA fragmentation. The study performed in the Goodenow laboratory (Steven Pomeroy) demonstrated that up to 1000 μM IDV is not toxic to PBMC. Although this concentration is higher than that reported in the literature[87, 87,91,102,113], it is important to note that the study in the Goodenow lab did not observe in tandem any other upstream or downstream markers of apoptosis, and that other indicators of apoptosis may have

been positive but undetected in the assay performed in the Goodenow laboratory. Taking these data into consideration, we can conclude from this pilot study ability to recapitulate published data in concert with the fact that 18 hour extracellular PI treatment confers peak intracellular PI levels.

Intracellular PI Studies: Parameters That Impact Intracellular PI Levels and Efflux

Multiple parameters can affect intracellular PI concentrations, including temperature, stimulation state of the cell, time, and concentration of serum in the extracellular media. Relative to baseline intracellular PI levels (immediately after 18 hour load of cells with PI at 37^{o}C), stimulation state and concentration of serum in the extracellular media can affect intracellular PI levels.[1,5,6,12,18,19,35,37,40, 43,56, 57, 64,106,109]

Keeping these factors in mind, we were able to design a study to assess the impact of temperature, time, and concentration of extracellular serum on intracellular PI levels. Our data demonstrate that efflux above limit of detection can occur when efflux is observed at 20^{o}C in unstimulated PBMC maintained in 2 % serum containing media. Keeping this fact in mind, the next step in the discussion of these data was to determine the best possible mechanism to test the hypothesis of this thesis.

Total Number of R5 Target Cells: Options and Alternative Methods

Although it is mathematically possible to obtain enough of the cells representing the R5 target cells within the lymphocyte population, other options exist that could present a more feasible mechanism to test the hypothesis of this thesis. Methods include use of efflux transporter +/- cell lines, RT-PCR, western blot analysis, and direct stain for efflux transporter expression. These experiments were not performed, although they, in concert with the preliminary data obtained in this thesis, provide an excellent foundation from which further

studies can be launched. Discussion regarding each method, its pitfalls, potential method of execution and analysis appear below.

Detained Analysis and Interpretation of Rh-123 Studies

Alternative method of Rh-123 assay was employed to assess p-gp activity within subsets of T lymphocytes. When analyzing Rh-123 data, multiple parameters must be considered. A summary of parameters, followed by discussion is as including: [1,2,4,5,6,9,12,18-22,35,37,40,42,44,51,56,57,60,63-66,68,70-73,76,77,81,93,94,98,100,104,106,107,109,112]

- Lipophilicity of drug
- Affinity of PI for p-gp
- Presence of intracellular proteins that may bind PI (and prevent efflux of PI)
- Ability of PI to modulate p-gp expression levels
- Activity state of p-gp during timeframe that PI efflux is being studied
- Combined effect of parameters 1-3 on Rh-123 efflux
- Presence of other efflux transporters and their potential effect on the system

Lipophilicity of Drug: Parameters for Analysis and Interpretation of Rh-123 Data

Of the ten FDA approved PI, NFV displays the greatest intracellular accumulation *in vitro* as assessed in cell lines and primary lymphocytes. The rank order of intracellular accumulation is NFV > RTV > IDV. These data were demonstrated in homogeneous p-gp expressing cell lines and in total PBMC [1,2, 19,21,56,57, 65]. Relative to the data reported in this thesis, consideration for lipophilicity of drugs tested (IDV and RTV) must occur. The data reported in this thesis demonstrate that R-Verapamil, a known substrate for p-gp, and inhibitor of Rh-123 efflux, inhibits nearly 80 % of Rh-123 efflux, whereas RTV and IDV inhibit ~ 30 % and ~ 80 %, respectively. Taken alone, these data would indicate that R-Verapamil and IDV display similar affinity for p-gp whereas RTV displays significantly less affinity for p-gp (and thus cannot out compete Rh-123 for p-gp mediated efflux).

Rank order of intracellular accumulation mirrors rank order of lipophilicity of PI, correlating higher lipophilicity with greater ability to enter cells and higher intracellular PI levels. No shuttle mechanism exists to transport substrate to p-gp for efflux. Therefore, the greater the intracellular concentration of drug, the higher the likelihood that the drug will come in contact with the efflux transporter and become effluxed. This principle holds true relative to ability to inhibit Rh-123 efflux. If more RTV is present inside the cell, then inhibition of Rh-123 will occur more readily than if little RTV was present intracellularly, as more drug is available to out compete Rh-123 for binding to p-gp.

Data reported in this thesis demonstrate that RTV inhibits significantly less Rh-123 relative to IDV (~30 % versus ~80 %). These data in the context of the correlation between intracellular accumulation and ability to inhibit Rh-123 efflux would indicate that IDV displays higher intracellular accumulation than RTV. As the Rh-123 assay cannot directly determine intracellular levels of drug, a direct correlation between intracellular PI levels and ability to inhibit Rh-123 efflux cannot be drawn to test the hypothesis for observed results.

It is important to understand that mechanisms responsible for data are multifactorial, and likely include factors that cannot be directly measured with the Rh-123 assay. A carefully designed set of experiments to elucidate mechanisms responsible for these data is necessary to test how hierarchical intracellular accumulation of PI affects the data reported in this thesis. Comprehensive discussion occurs in the alternative methods section of this thesis relative to approaches to elucidate mechanisms responsible for data, and their relationship to the factors bulleted above.

Affinity of PI for p-gp: Analysis and Interpretation of Rh-123 Data

Drugs bind with differential affinity to efflux transporters. Multiple studies have demonstrated that Rh-123 is a substrate for p-gp [68], however differential affinity of PI within

lymphocytes, and in subsets of lymphocytes, is largely undefined. Although RTV displays greater intracellular accumulation than IDV [44], RTV may display lower affinity for p-gp than IDV, rendering RTV less effective in competitive inhibition of Rh-123 efflux. In addition, affinity of RTV and IDV for other efflux transporters relative to their affinities for p-gp, and relative to each other, is not defined. If RTV displays higher affinity for MRP1, for example, relative to p-gp, then RTV could be primarily and preferentially effluxed from the cell via a p-gp independent mechanism. Rh-123 assay can indirectly detect efflux of PI via p-gp dependent mechanisms, but not other efflux transporters. The contribution of other efflux transporter expression, activity, and affinity of PI for these transporters cannot be detected within the confines of the experiment conducted.

Therefore, in the context of the data reported in this thesis, it is difficult to explain one specific mechanism responsible for the results. A carefully designed set of experiments to elucidate mechanisms responsible for these data is necessary to test how affinity of PI affects the data reported in this thesis. Comprehensive discussion occurs in the alternative methods section of this thesis relative to approaches to elucidate mechanisms responsible for data.

Presence of Intracellular Proteins: Parameters for Analysis and Interpretation of Rh-123 Data

To understand the mechanism responsible for our data, other parameters must be considered. PI are designed for therapeutic function of inhibiting HIV-1 Protease, which is an aspartic protease. PI are have been shown to bind and interact with cellular proteases, most notably caspases (cysteine proteases). [77,79,88,102,103] PI have also been implicated as inhibitors of cellular proteasome function.[89,90] It is hypothesized, but currently undefined, that PI binding/interaction with intracellular proteins is not limited to caspases or proteasomes.

Upon binding, PI are subsequently unavailable to bind to efflux transporters, and are often physically immobilized, preventing their ability to bind to efflux transporters [1,18,19,40,70]. Whether RTV and IDV bind to different proteins, bind to proteins with different affinities, or whether these proteins display differential expression levels within subsets of cells, is unknown. Our data observed ability of RTV, IDV, or R-Verapamil ability to inhibit Rh-123 efflux in the subpopulation of lymphocytes representing the high p-gp expressing R5 HIV-1 target cells. These data may or may not hold true when observed in a total lymphocyte population, within other subsets of lymphocytes, or across multiple donors. The conclusion is multi-fold:

- RTV may bind to proteins that IDV does not, thus impeding its ability to inhibit Rh-123 efflux
- Protein expression levels that RTV or IDV may bind to could be different in the population of cells observed relative to other cell populations. RTV and IDV may display different percent inhibition of Rh-123 in other subsets of cells or in a total population of PBMC.
- These experiments must be repeated in multiple donors to confirm results, and coupled with tandem experiments to elucidate mechanisms responsible for results (discussed in alternative methods section of this thesis).

Further analysis and/or experiments must be performed to answer these questions. Assessing percent inhibition of Rh-123 efflux by RTV and IDV in other subsets of lymphocytes and in total PBMC would determine if RTV inhibits a greater percentage of Rh-123 efflux within different cell populations. Testing ability of IDV and RTV to inhibit Rh-123 efflux in homogeneous cell lines (and/or total PBMC) presents a follow up experiment to test ability recapitulate published data, ruling out technical error as mechanism for results.

Modulation of p-gp by PI: Parameters for Analysis and Interpretation of Rh-123 Data

NFV, but not other PI or R-Verapamil (p-gp inhibitor) increase p-gp and MRP1 expression *in vitro* [20,43]. As p-gp expression indirectly correlates with p-gp activity [68], it is important to understand the relationship between drug, efflux transporter expression, and Rh-123 efflux.

Increased p-gp expression in a cell will result in more rapid efflux of Rh-123 relative to cells with lower p-gp expression. IDV and RTV do not increase efflux p-gp expression [20,43], therefore presence of these drugs cannot account for more rapid efflux of Rh-123 as a function of increased p-gp expression. IDV and RTV do not increase MRP1 transporter expression, however effect of these drugs on MRP2, MRP4, MRP5, or BCRP is unknown. If PI increase expression of these efflux transporters, then the PI will consequently be effluxed more rapidly. This effect would be undetectable within the confines of the Rh-123 assay, as it observes only p-gp mediated efflux and does not consider efflux by other mechanisms.

As no data is currently available to support or deny the idea that PI may modulate MRP2, MRP4, MRP5, or BCRP, pilot experiments could be performed to address and fill current gaps in knowledge. Western blot, RT-PCR, and direct staining for efflux transporters with specific flurochrome conjugated mAb for cells treated with or without PI over time, with appropriate controls presents options for execution of these pilot studies.

Activity State of p-gp: Parameters for Analysis and Interpretation of Rh-123 Data

In all cases, presence of PI within the binding pocket of the efflux transporter activates the efflux transporter, allowing for efflux of PI via the ATP dependent mechanism. Efflux transporters remain in a basal, non-active state until substrate-binding pocket is engaged by substrate. As no shuttle mechanism exists to transport substrate to efflux transporter (subsequently conferring activation of the transporter), activation occurs when substrate is in close physical proximity to substrate binding pocket.[5,18,19,70] Points above addressed binding of PI to intracellular proteins and inability of PI to bind to substrate binding pocket once bound to intracellular proteins. As limited data exist regarding the profile of intracellular proteins to which PI bind (other than caspases and proteasomes), and with what affinity, it is difficult to determine what, if any, role this protein/PI interaction may have on ability of PI to interact with

p-gp and subsequently confer activation. Independent of these gaps in knowledge, we have demonstrate that IDV, RTV, and R-Verapamil inhibit Rh-123 efflux, something that could not occur if drugs were completely bound to intracellular proteins. The statistical significance of these data is unknown, as n=1 and no technical replicates were performed within the donor tested. These experiments must be repeated, and followed up as described below to confirm/validate results.

Pitfalls of Rh-123 Study

Although Rh-123 provides an excellent mechanism to observe p-gp activity within subsets of cells, the assay displays shortcomings and pitfalls. These shortcomings can be addressed by performing a comprehensive and carefully planned set of follow up experiments to elucidate specific mechanisms responsible for Rh-123 efflux data. A list of shortcomings and pitfalls are summarized below followed by discussion for potential execution of experiments to rectify each shortcoming/pitfall.

- Assay is indirect measure of p-gp expression (observes p-gp activity)
- P-gp activity correlates with, but is not always directly proportional with, p-gp expression
- Requires further follow-up experiments with direct assessments of p-gp expression to confirm hypotheses about p-gp expression obtained from Rh-123 p-gp activity studies
- Assay cannot discern mechanism responsible for Rh-123 efflux: (are results a function of p-gp activity alone or potential modulation of p-gp expression levels (or other efflux transporter levels) that may occur as a function of presence of substrate [as occurs with NFV]) ?
- Rh-123 can only indirectly assess intracellular bioavailability of PI. It is twice removed from intracellular PI levels (p-gp activity is indirectly related to p-gp expression, which correlates with, but is not a direct measure of, intracellular PI levels)
- Rh-123 assay is specific for p-gp and cannot be used to determine activity of other efflux transporters for which PI are substrates, or their impact on efflux of PI by p-gp

Alternative Methods to Assess p-gp Activity and Expression

Many assays are commonly used to directly assess not only p-gp expression, but intracellular bioavailability of PI within cells.[1, 19-22,30,41-44,56,57,59,60,63,65,66,68,71,73,77,84,93,97,98,106,107,112] When performed in tandem, these experiments can establish relationships between efflux transporter expression and intracellular levels of drug.

In the study performed in this thesis, follow up experiments can be performed to confirm and/or test the hypothesis that differential p-gp activity is an indicator of differential p-gp expression, which directly correlates with intracellular PI levels. This study sought to determine the mechanism(s) responsible for significantly higher IC_{50} in R5 versus X4 using viruses in T lymphocytes. Relative to the initial question posed, additional follow up experiments to directly assess efflux transporter expression in these subsets as well as intracellular PI levels within these subsets could be performed and present an excellent starting point from which to launch further studies.

Follow Up to Rh-123: Direct Assessment of Efflux Transporter Expression

Flow cytometry presents the most feasible method for directly assessing p-gp expression. Flurochrome conjugated mAb specific for each of the six efflux transporters for which PI are substrates (p-gp, MRP1, MRP2, MRP4, MRP5, BCRP) are commercially available and have been demonstrated in numerous studies to provide accurate assessment of efflux transporter expression as confirmed with comparative analysis of analogous cells subjected to western blot and RT-PCR analysis. [42,66,68,71,72]

Performing p-gp expression studies will allow for confirmation of p-gp expression that is hypothesized from p-gp activity profile obtained with Rh-123 data. The mechanisms to directly assess intracellular PI levels exist: [29,42,62]

- HPLC and tandem mass spectrometry conducted on cellular lysates of cells previously treated with PI
- Radio-labeled PI

In the case of this study, pre sorting of cells must occur for HPLC and mass spectrometry, or radio-labeled PI to be employed within subsets of lymphocytes, and presents potential problems with total cell number and constraints. In the context of the data obtained in the thesis, an excellent follow up to confirm hypothesis of differential p-gp expression in lymphocyte subsets could be flow cytometry with mAb specific for p-gp with gating on specific lymphocyte subsets. In addition, the data set could be expanded to encompass all efflux transporters for which PI are substrates, allowing for a comprehensive analysis of relationship between efflux transporters for which PI are substrates, and expression within X4 and R5 HIV-1 target cells within the lymphocyte population.

Pilot experiments to assess appropriate combination of fluorochromes and combinations, as well as reproducibility across donors must first be conducted to achieve the end goal. In addition, use of cell line with known levels of efflux transporter expression could be employed as a preliminary study to test inter and intra assay variability, and ability to observe efflux transporters within the parameters of the newly designed experiment.

Direct assessment of efflux transporter expression presents an excellent foundation to assess levels of all know efflux transporters for which PI are substrates, within the X4 and R5 HIV-1 target cells. It is important to note that performing stain for efflux transporter expression would not fully elucidate the mechanisms responsible for higher IC_{50} in R5 versus X4 T lymphocytes. Instead, these data would provide an excellent foundation from which to launch further studies to elucidate the complex relationship between drug, efflux transporter activity, expression, and intracellular accumulation of drug.

Relationship Between p-gp Activity and IC_{50}

The questions posed above culminated in the finding that a subset of lymphocytes that are R5+/CD4+/CD45RA-/CD45RO+ displayed p-gp levels comparable to the highest known p-gp levels in any human cell, NK cells[67]. Cells expressing CD4 and R5 are targets for HIV-1 infection by an R5 using virus. The population of cells displaying high p-gp activity represents one of the populations for R5 infection within the lymphocyte population, demonstrating a link between p-gp activity in the R5+/CD4+/CD45RA/CD45RO+ cells and the requirement for more drug in viruses targeted to R5 expressing T lymphocytes.

Although PI are substrates for other efflux transporters, and p-gp is not the sole determining factor for efflux and bioavailability,[1, 2,4,5,6,9,12,18-22,35,37,40,42,44,51,56,57,60,63-66,68,70-73,76,77,81,93,94,98,100,104,106,107,109,112] these data demonstrate that p-gp activity is higher in a subset of the R5 target cells relative to cells representing the X4 HIV-1 targets. As p-gp activity is linked directly to intracellular bioavailability of PI, these data demonstrate a conclusive link between p-gp activity and differences in IC_{50} for otherwise identical viruses targeted to R5 versus X4 expressing T lymphocytes. Follow up experiments must be performed to confirm this hypothesis, as described in the alternative methods section of this thesis, in concert with repetition of the reported data in multiple independent donors.

Relationship Between Fitness, IC_{50}, and Target Cells

The primary goal of the thesis centers around significant differences in IC_{50} for otherwise identical viruses targeted to R5 versus X4 expressing T lymphocytes. It is important to relate the fitness of these viruses to IC_{50}, as this relationship further implicates difference in target cell as a modulator of not only IC_{50} values but also replicative capacity.

Previous studies in our laboratory demonstrated that X4 using recombinant viruses containing posttherapy PI^{res} *gag-pol* fragment display a significant reduction in p24 production

relative to otherwise identical recombinant viruses with pretherapy PI^{sen} *gag-pol* fragment whereas pre and posttherapy R5 recombinant viruses replicate to similar levels. These studies were conducted with otherwise identical viruses targeted to X4 or R5 expressing T lymphocytes, implicating differences in target cell as a modulator or differential replicative capacity. Although published findings report that R5 viruses display a fitness advantage relative to X4, these reports did not use recombinant viruses, nor was there a direct comparison of LAIX4 envelope with JRFL V1-V5 envelope, which were used in our studies. [8,17, 96]

Fitness studies in our lab were expanded to address differences in target cell relative to IC_{50}. It was determined that significantly more drug (188 fold for IDV, 141 fold for RTV) is required to inhibit viral replication for otherwise identical viruses targeted to R5 versus X4 T lymphocytes. As the only difference for these viruses are the target cell, this finding suggested that differences in the cells, not virus, are responsible for the outcome.

Relating data from our laboratory to published findings from other groups is important in determining the full impact of data obtained from this thesis. Schuitemaker[97] *et al* reported that intracellular HIV-1 RNA and DNA was significantly lower in cells with high-p-gp activity relative to low p-gp activity (as assessed by Rh-123 assay), thus concluding that the potential efflux function of p-gp on PIs may be clinically less relevant than the effect of p-gp on intracellular HIV-1 replication. To correlate these data to findings from this thesis, consideration for target cell population must occur. The group used Rh-123 assay in concert with CD45RO stain to delineate two groups: RO+/high-p-gp activity, and RO-/low-p-gp activity cells. Gating solely on CD45RO is not a surrogate marker for X4 versus R5 HIV-1 target cells within the lymphocyte population. As no gating or stain for CD45RA, CCR5, or CXCR4 was performed, the analysis conducted by Schuitemaker is focusing upon two populations of cells which may co-

express varying levels and combinations of CXCR4 and or CCR5. In addition, our data demonstrate that ~30 % of CD4+ lymphocytes co-express CD45RA and CD45RO, demonstrating that gate on CD45RO does not eliminate inclusion of CD45RA. Although Schuitemaker's data presents an interesting correlation between intracellular HIV-1 RNA and DNA and p-gp activity, it is important to note that this correlation is drawn based on a population of cells that is different from the cells tested in this thesis.

Despite different target cell analysis, one cannot discount either the findings from this thesis or Schuitemaker's data. Instead, the data from this thesis, in concert with Schuitemaker's findings, present an excellent foundation from which to launch further studies to confirm the hypothesis of this thesis, and conduct *in vivo* studies to observe p-gp activity and viral RNA or DNA within the R5 and X4 HIV-1 lymphocyte targets.

Relationship of Results to Therapy Outcome and Disease Progression

Findings from this thesis impact multiple interactions between drug, host, and virus. Although differences in bioavailability of PI within lymphocyte subsets explains significantly higher IC_{50} for otherwise identical viruses targeted to R5 versus X4 T-lymphocytes, these data impact other determinants in HIV-1 infection. This study draws a link between differential p-gp activity in R5 versus X4 T lymphocytes, and may indicate that differences between these target cells is not limited to p-gp activity. Furthermore, findings from this thesis also provided a foundation from which to launch further studies to determine how PI impact HIV-1 target cells, and to determine how these events relate to therapy outcome and disease progression.

Impact of PI Independent of Antiviral Effect: Relationship to Therapy Outcome

When treating HIV infected patients, the goal is to delay progression to AIDS by controlling viral replication and preventing or reversing immune deficiency [29,34-36]. However, in a significant percentage of children and adolescents, immune reconstitution is achieved but not

concomitant with decrease in viral burden, implicating a role for ART, and specifically PI, in immune reconstitution independent of its antiviral effect [13, 23-27, 39, 43, 46, 61, 83]. Sentinel events that favor immune reconstitution and decreased susceptibility to HIV-1 infection have been observed upon addition of PI either *in vivo* or *in vitro*. Such extravirologic properties of PI include modulation of antigen presentation, cell cycle arrest, cell proliferation, ABC efflux transporter activity, lipoprotein metabolism, dendritic cell maturation, and inhibition of spontaneous and activation induced apoptosis of both HIV-1 infected and uninfected T lymphocytes [2,4,12,15,21,23,38,44,52,60,62,78,79,87-91,102,103,113-115]

Immune reconstitution is highly favorable for patient prognosis and leads to delayed progression to AIDS [29,34-36]. ART induced discordant responses are multifactorial and involve a complex interplay between phenotypic properties of the virus, ART effect and interaction with host cells, and the ability of the immune system to control viral replication in the presence of ART. The effect of PI on the immune response independent of its antiviral effect, and the implications of differential effect of PI within subsets of HIV-1 target cells and in other cells, constitutes a complex set of questions relating to therapy outcome and progression to AIDS.

Relationship Between Efflux Kinetics, Peak Intracellular PI Levels, and Cell Cycle Studies

Mechanisms correlating failure of therapy with R5 emergence are not fully understood. This foundation of this study sought determine if efflux kinetics and peak intracellular PI levels in subsets of HIV-1 target cells could be responsible for requirement of significantly more drug required to inhibit viral replication for otherwise identical viruses targeted to R5 versus X4 T-lymphocytes. This correlation relates not only to the mechanism responsible for requirement of significantly more drug for the R5 subset, but also relates to therapy outcome for HIV-1 infected individuals, and may explain emergence and persistence of R5 infection. In addition, these findings in concert with published data demonstrating effect of PI on cells independent of their

antiviral effect, provide a base from which to launch further studies to determine additional impact of PI on cells independent of their antiviral effect.

The appendix of this thesis addresses a pilot study designed to determine the effect of IDV, RTV, and NFV on cell cycle progression in total PBMC. It was determined that IDV, RTV, and NFV induce a G1 block in a dose dependent manner. This study was conducted with the idea that further studies could be explored to determine the mechanisms responsible for G1 block, to relate these data to potential differences in subsets of HIV-1 target cells, and to draw a correlation between these data and therapy outcome for patients receiving a PI containing regimen. Further studies were not conducted on this project, however findings present an excellent foundation for further experiments.

Significance and Implications for Differential p-gp Activity in CD4+/CD45RA-/CD45RO+/CCR5+ T lymphocytes

These data may have implications for *in vivo* replication dynamics, therapy outcome, persistence of R5 infection, and viral reservoirs for drug resistant viruses emerging under the selective pressure of suboptimal levels of drug. Suboptimal levels of ART is an ongoing problem in treating HIV-1 infection and presents obstacles including emergence of resistant mutations and clearance of virus from infected cells with low bioavailability [1,6,9,10,21,40,43,76,77,81]. Understanding dynamics of virus/host/drug interaction and identifying mechanisms for decreased bioavailability of ART can lead to design of novel therapeutics to increase intracellular concentrations of drug.

APPENDIX A
IMPACT OF HIV-1 PROTEASE INHIBITORS ON CELL CYCLE PROGRESSION IN PBMC

Introduction

Previous studies have established that PI have an effect on cells independent of their antiviral activity against HIV-1. Sentinel events that favor immune reconstitution and decreased susceptibility to HIV-1 infection have been observed. Such extravirologic properties of PI include modulation of antigen presentation, cell cycle arrest, cell proliferation, ABC efflux transporter activity, lipoprotein metabolism, dendritic cell maturation, and inhibition of spontaneous and activation induced apoptosis of both HIV-1 infected and uninfected T lymphocytes. [2,4,12,15,21,23,38,44,52—60,62,78,79,87-91,102,103,113-115] The objective of the experiments in this appendix was to discover a role for PI in modulating cells that may influence virus/host cell interactions and explain a role for PI in impacting therapy outcome independent of its antiviral effect.

A unique paradigm is observed in 25-40 % of children and adolescents who, following initiation of a PI containing regimen, exhibit a VF/IS discordant response (sustained reconstitution of T cell immunity despite transient viral suppression and subsequent rebound to levels which predict disease progression). This discordant response is not determined solely by host factors alone or simple genetic changes in the viral genome. ART induced discordant responses are multifactorial and involve a complex interplay between phenotypic properties of the virus, ART effect and interaction with host cells, and the ability of the immune system to control viral replication in the presence of ART. [39, 100, 116,118]

The experiments in this appendix were based on one specific facet of the VF/IS discordant response: effect of PI on cells independent of their antiviral effect. The experiments were based on the hypothesis that PI modulation of cell cycle progression in lymphocytes may

be a determinant in therapy outcome. Cells that are actively progressing through the cell cycle are significantly more susceptible to HIV-1 infection relative to quiescent, non-cycling cells. If PI can induce cell cycle arrest in HIV-1 target cells, this would render these cells significantly less susceptible to HIV-1 infection. This hypothesis could represent a model where PI favorably impact cells independent of their antiviral effect.

Pahwa, 2001 *et al*, reported that IDV inhibits cell cycle progression via a G1 block in CD4+ T-lymphocytes in a dose dependent manner [23]. The study in this appendix sought to determine if other PI (RTV and NFV) modulate cell cycle progression in lymphocytes, and to relate these data to therapy outcome reported for patients receiving a RTV or NFV containing regimen.

Significance

When treating HIV infected patients, the goal is to delay progression to AIDS by controlling viral replication and preventing or reversing immune deficiency. However, in a significant percentage of children and adolescents, immune reconstitution is achieved but not concomitant with decrease in viral burden, implicating a role for ART, and specifically PI, in immune reconstitution independent of its antiviral effect. Immune reconstitution is highly favorable for patient prognosis and leads to delayed progression to AIDS. [39, 100, 116,118] Determining mechanisms responsible for favorable effect of PI on immune reconstitution independent of its antiviral effect may lead to design of novel therapeutics, which exploit host immune response.

Methods for PHA or Anti-CD3mAb Stimulation

The studies in this appendix were conducted with the intent of determining effect of PI on cell cycle progression in PBMC and correlating these data with fitness and IC_{50} data previously obtained in the Goodenow laboratory. All fitness and IC_{50} studies were conducted on PHA

stimulated PBMC, thus PHA as a means of stimulation to induce cell cycle progression was first studied. Published data from Pahwa, *et al* determined that IDV induced a G1 block in cell cycle progression but did so using anti-CD3mAb stimulated lymphocytes. This group subjected the lymphocytes to PI for 18 hours prior to 48-hour stimulation with anti-CD3mAb in the absence of PI. Therefore, in the context of the original intent or these studies and the published data, both PHA and anti-CD3mAb was tested for its ability to induce cell cycle progression in PBMC.

Cryopreserved PBMC isolated as described previously from one donor were cultured at a concentration of $1.0x10^6$ c/mL for 18 hours at 37°C in RPMI 1640 supplemented with 30 U/mL human interleukin-2, 2 mM L-glutamine and 100 U of penicillin per mL, 100 g of streptomycin per mL, and 2% heat- inactivated fetal calf serum. After 18 hours, PBMC were treated with 0.5μg/mL anti-CD3mAb (Pharmingen) for 48 hours, or 0, 10, 25, 50, or 100 μg/mL PHA for 48 or 72 hours at 37°C. Cells were then centrifuged in 15 mL conical tubes and centrifuged at 1000 rpm for 10 minutes. Residual media was removed, and cell pellets were subjected to three washings in ice cold 1X PBS at 1000 rpm for 10 minutes. After washings, cell pellets were fixed in 70% ethanol for 1 hour and incubated with Propidium Iodide (Invitrogen) (50 g/mL) and RNAse (Invitrogen) (100 g/mL). Samples were kept in the dark at 4°C for 30 minutes and immediately analyzed for cell-cycle profile using FACS Vantage Flow Cytometer (Beckman Dickinson), Mod-Fit Software (Verify Software, Inc.).

Methods for Assessment of PI Effect on Cell Cycle Progression

Cryopreserved PBMC isolated as described previously from one donor were cultured at a concentration of $1.0x10^6$ c/mL for 18 hours at 37°C with 0, 10, 25, 50, or 100 μM IDV, RTV, or NFV in RPMI 1640 supplemented with 30 U/mL human interleukin-2, 2 mM L-glutamine and 100 U of penicillin per mL, 100 g of streptomycin per mL, and 2% heat- inactivated fetal calf

serum. After 18 hours, PBMC were treated with 0.5μg/mL anti-CD3mAb (Pharmingen) in drug free media (figure A-2)_. Technical triplicates were prepared for each sample.

After 48-hour anti-CD3mAb treatment, cells were then centrifuged in 15 mL conical tubes and centrifuged at 1000 rpm for 10 minutes. Residual media was removed, and cell pellets were subjected to three washings in ice cold 1X PBS at 1000 rpm for 10 minutes. After washings, cell pellets were fixed in 70% ethanol for 1 hour and incubated with Propidium Iodide (Invitrogen) (50 g/mL) and RNAse (Invitrogen) (100 g/mL). Samples were kept in the dark at 4°C for 30 minutes and immediately analyzed for cell-cycle profile using FACS Vantage Flow Cytometer (Beckman Dickinson), Mod-Fit Software (Verify Software, Inc.), and Sigma Stat 3.0 (SEM based on technical triplicates) (Figure A-1).

Results

For quiescent, non-Anti-CD3mAb, non-PHA stimulated PBMC, ~ 85 % of PBMC were in G1, ~ 15 % of PBMC were in S, and no PBMC were in G2/M phase. For Anti-CD3mAb stimulated PBMC, ~ 50 % of cells were in G1 whereas the other half of cells were in S or G2/M (Figure A-3, A-4). For PBMC treated with PHA, percent of cells in Go/G1 ranged from 25-50 % independent of time exposed to PHA or concentration of PHA applied (Figure A- 1).

IDV, RTV, and NFV inhibited cell cycle progression in a dose-dependent manner,. Nearly 80 % of PBMC arrested in G1 upon exposure to 100 μM IDV, whereas nearly 90 % of cells were arrested in G1 upon exposure to 50 μM RTV or NFV, with the remaining cells in S phase (Figure A-3, A-4).

Discussion

This study demonstrated that IDV, RTV, and NFV inhibit cell cycle progression in PBMC via a G1 block in a dose dependent manner. Specifically, 50 μM RTV and NFV induced

a G1 block for a significant percentage of cells ($\geq$ 80 %) and IDV induced a G1 arrest upon 100 μM treatment. *In vivo*, the peak plasma level of extracellular PI that are non-plasma protein bound and available to traverse the lipid bilayer and enter cells is ~ 8.0 μM for IDV, 11.0 μM for RTV, and ~ 3.0 μM for NFV. The concentration required to induce a G1 block in total PBMC *in vitro* is significantly higher (~ 50 μM). This study was conducted on a heterogeneous population of cells including monocytes, NK cells, and multiple subsets of T-lymphocytes. These cells display significantly different levels of efflux transporter expression and activity. These experiments answered a critical question relative to PI effect on cell cycle progression in PBMC, however analysis of physiologically relevant concentrations of drug in specific subsets of lymphocytes, or HIV-1 target cells within the lymphocyte population cannot be determined from this pilot experiment, and must be considered in further experiments.

We can determine that a concentration of PI that is not physiologically relevant to *in vivo* pharmacokinetics results in a G1 block when observing the total PBMC population. Published literature and findings from the objectives in the parent thesis demonstrate differential efflux transporter activity and expression, coupled with significant differences in IC_{50} for otherwise identical viruses targeted to R5 versus X4 T-lymphocytes. The hypothesis is that physiologically relevant concentrations of PI (3-11 μM PI) may induce a G1 block in particular subsets of lymphocytes which display lower efflux transporter activity and expression but not in subsets which display high efflux transporter activity and expression (where high is defined as similar to NK cells, which display highest p-gp activity of any known human cell) [68].

Relative to the IC_{50} studies and the Rh-123 studies, we can hypothesize that subsets of lymphocytes that may arrest in G1 upon exposure to physiologically relevant concentrations of PI include the X4 HIV-1 target cells, and that the R5 HIV-1 target cells (in the lymphocyte

population) represent cells that would likely undergo a G1 arrest. No follow up experiments to confirm these hypotheses were performed, however these ideas present an excellent foundation for future studies to answer these questions.

Ability to test the hypothesis relies on ability to observe cell cycle progression within subsets of T-lymphocytes, and presents technical limitations on two fronts: 1) ability to separate enough of the R5 HIV-1 target cells, and the financial constraints associated with this task, and 2) inability to observe receptor expression profiles in cells permeablized to allow for DNA stain (either Propidium Iodide or 7-Amino-actinomycin D (7AAD) to enter cells (Figure A-5). Propidium Iodide is a fluorescent vital dye that stains DNA. It does not cross the PM of cells that are viable or in the early stages of apoptosis because they maintain PM integrity. Vital cells must be permeablized via exposure to 70 % ethanol, allowing for the dye to enter cells and subsequently bind to DNA. 7AAD, an alternative to Propidium Iodide, does not readily enter vital cells, and manufacturer protocol recommends permeablization of cells with ethanol, which precludes concomitant receptor expression profile studies (Figure A-4).

Administration of a PI containing regimen *in vivo* is often associated with CD4 T cell reconstitution [46,100,108]. These results may not be solely determined by the antiviral activity of PI, as many reports of favorable effects of PI on immunity have been reported independent of antiviral activity.[2,4,12,15,21,23,38,44,5260,62,78,79,87-91,102,103,113-115]

Here, a pilot study was conducted, where findings may indicate that IDV, RTV, and NFV may prolong cell survival either directly or indirectly via cell cycle arrest. In patients receiving a PI containing regimen, PI modulation of cellular functions may favorably impact CD4 T cell immunity and immunologic outcome independent of its antiviral effect. Although no experiments were conducted to answer these questions, the pilot experiments in this appendix

present an excellent foundation from which to launch further studies to determine effect of PI on cells independent of their antiviral effect. Understanding specific mechanisms responsible for these effects could lead to design of novel therapeutics, which directly affect these events.

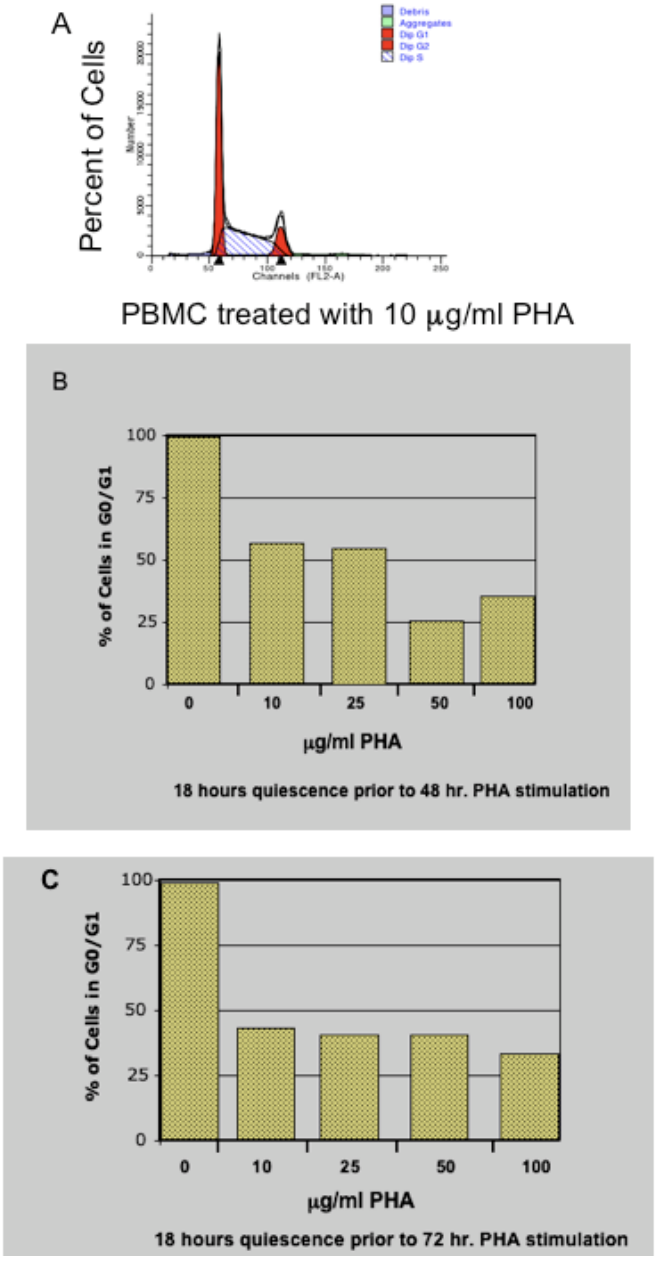


Figure A-1. Effect of PHA on cell cycle progression in PBMC. (A) Representative chromatograph of cell cycle progression for PHA treated PBMC. (B) PBMC treated with 0, 10, 25, 50, or 100 mg/mL PHA for 48 hours, or (C) 72 hours prior to 24 hour quiescence . Non PHA treated PBMC are 100 % in Go/G1 phase whereas PHA treated PBMC are 25-50 % in Go/G1 phase (B, C). Data are representative of one donor tested without replicates.

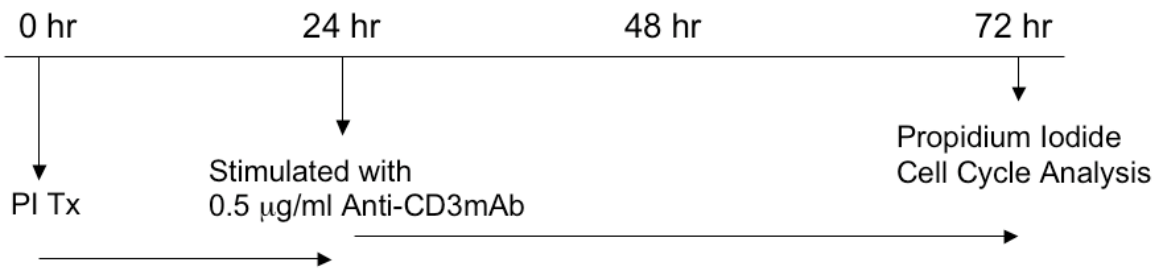


Figure A-2. Experimental design for cell cycle PI study in PBMC. After PBMC isolation, PBMC were resuspended in media containing 2 % fetal bovine serum and 0, 10, 25, 50, or 100 μM IDV, RTV, or NFV for 24 hours prior to stimulation with 0.5μg/mL anti-CD3 mAb in drug free media for 48 hours. At 72 hours post PI treatment, Propidium Iodide cell cycle analysis was performed. Cells were treated with 0, 10, 25, 50, or 100 μM IDV, RTV, or NFV.

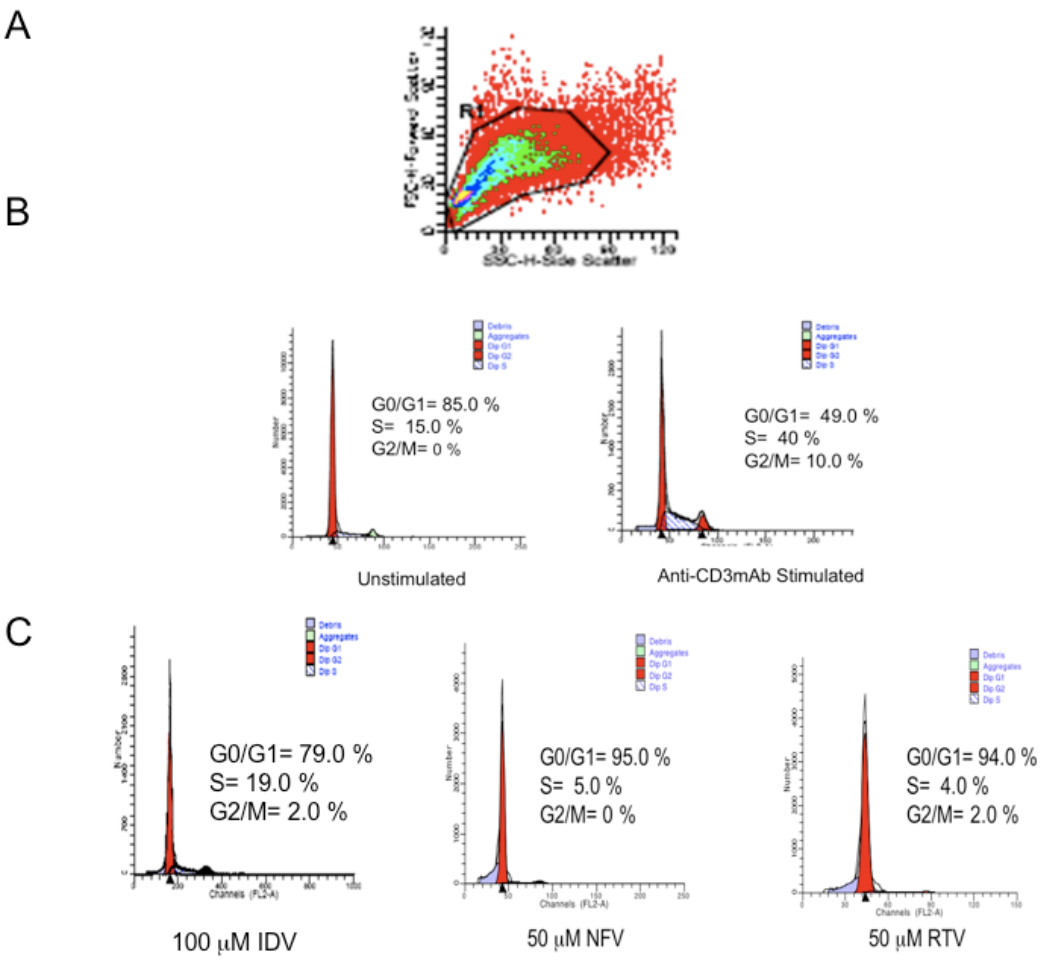


Figure A-3. Effect of PI on cell cycle progression in PBMC. Gate was drawn around lymphocyte population (A). Unstimulated PBMC were 85 % in G1 and 15 % in S phase and anti-CD3mAb stimulated PBMC were ~50 % in G1, 40 % in S, and 10 % in G2/M (B). PBMC treated with 100 μM IDV were ~ 80 % in G1, 19 % in S, and 2 % in G2/M. PBMC treated with 50 μM NFV were 95 % G1, 5 % S, and0 % in G2/M. PBMC treated with 50 μM RTV were 94 % in G1, 4 % in S, and 2 % in G2/M. Data are representative of n=1 with technical triplicates.

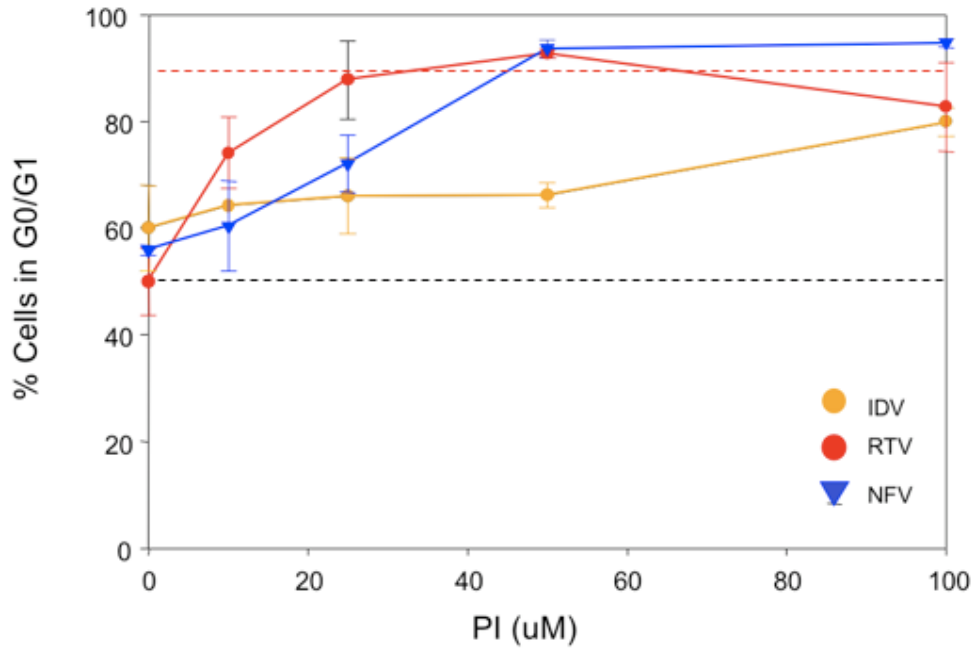


Figure A-4. Effect of IDV, RTV, and NFV on cell cycle progression in PBMC. IDV, RTV, and NFV inhibited cell cycle progression in a dose dependent manner via a G1 block. IDV inhibited arrested nearly 80 % of cells in G1 upon 100 μM treatment (yellow), and RTV and NFV arrested ~ 95 % of cells in G1 upon 50 μM treatment (red and blue, respectively). Red dotted line indicates percentage of quiescent cells in G0/G1 and black dotted line indicates percentage of anti-CD3mAb stimulated cells in G0/G1. Data are n=1 with technical triplicates. Error bars represent SEM.

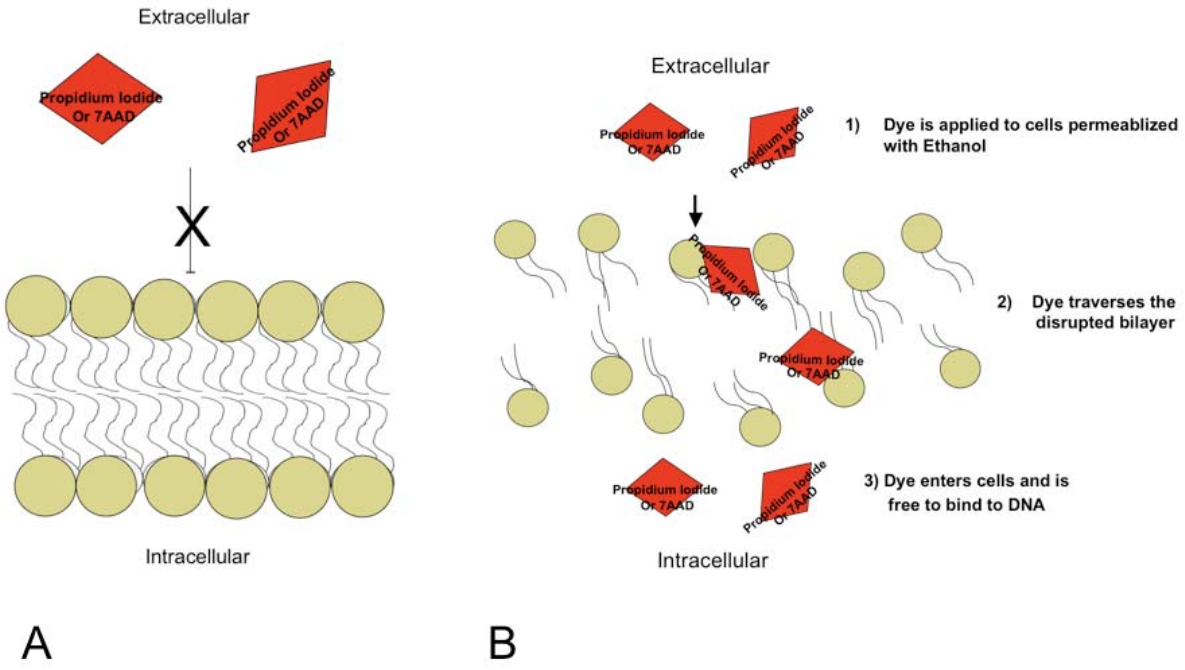


Figure A-5. Pitfalls for Propidium Iodide or 7AAD in concert with receptor expression stain. Vital cells possess an intact lipid bilayer, which cannot be penetrated by Propidium Iodide or 7AAD (A) and intact receptors (not shown). Cells permeablized by exposure to ethanol results in a non-intact lipid bilayer, which can be penetrated by Propidium Iodide or 7AAD (B), and disrupted receptor expression (not shown).

APPENDIX B
RAW DATA FOR RECEPTOR EXPRESSION AND INTRACELLULAR PI STUDIES

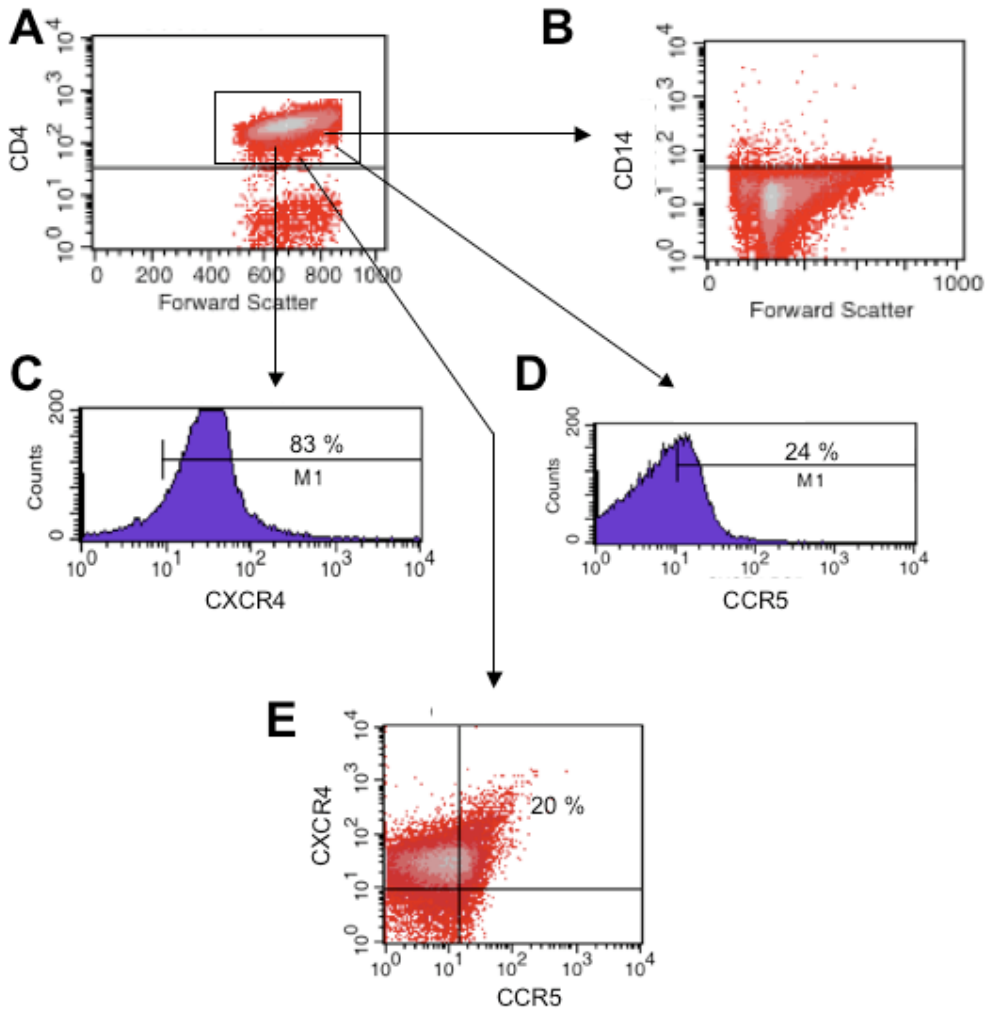


Figure B-1. Receptor expression profile for CD4 enriched lymphocytes, donor B. CD4 enriched population was gated on CD4 expression (A). ~ 80 % of enriched cells were CD4 positive (A). CD4 positive lymphocytes were then subjected to gate on CD14 (A). None of the CD4 enriched cells expressed CD14 (monocytes) (B). CD4 positive lymphocytes were subjected to gate on CXCR4 (C) or CCR5 (D), or gate on CXCR4+/CCR5+ (E). Percent of CD4/CXCR4 cells is ~ 80 % of enriched population, and percent of CD4/CCR5 cells is ~ 20 % of enriched population. Percent of CD4+/CXCR4+/CCR5+ T lymphocytes is ~ 20 %.

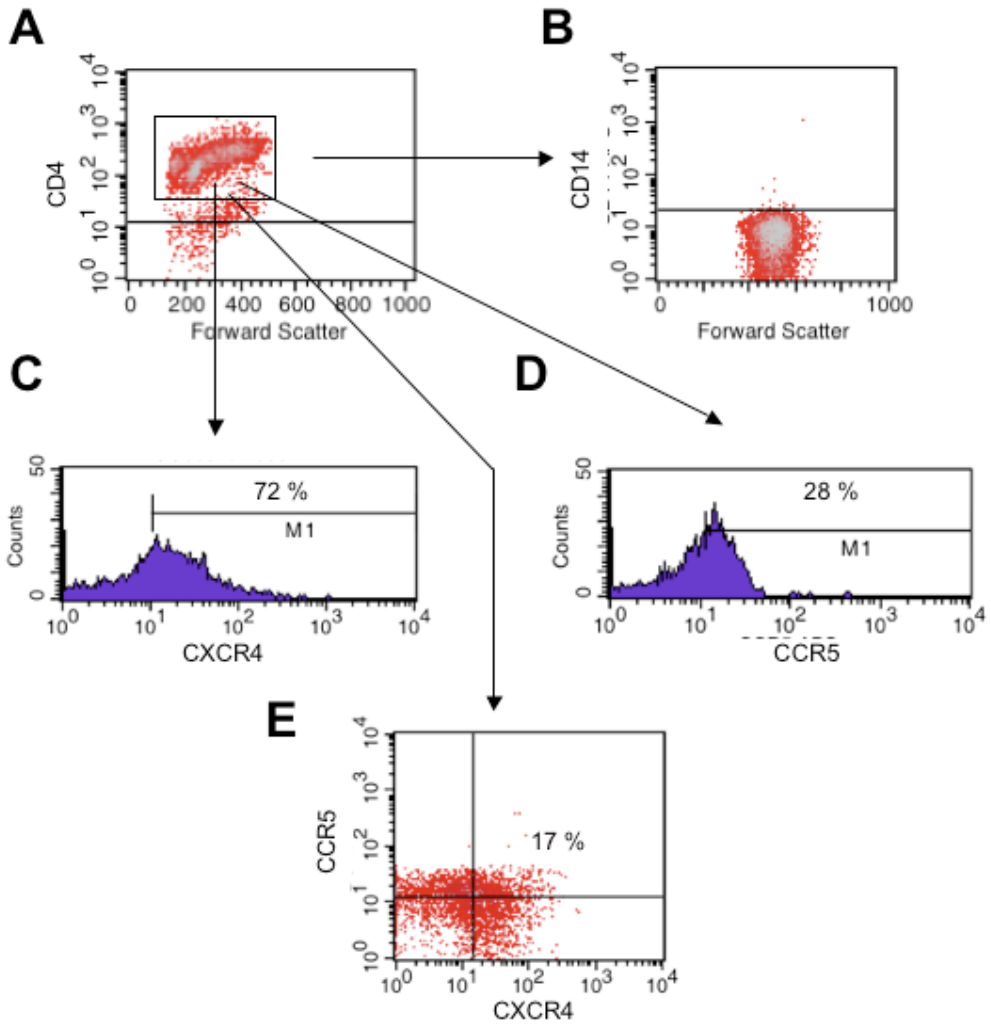


Figure B-2. Receptor expression profile for CD4 enriched lymphocytes, donor C. CD4 enriched population was gated on CD4 expression (A). ~ 80 % of enriched cells were CD4 positive (A). CD4 positive lymphocytes were then subjected to gate on CD14 (B). None of the CD4 enriched cells expressed CD14 (monocytes) (B). CD4 positive lymphocytes were subjected to gate on CXCR4 (C) or CCR5 (D), or gate on CXCR4+/CCR5+ (E). Percent of CD4/CXCR4 cells is ~ 72 % of enriched population, and percent of CD4/CCR5 cells is ~ 25 % of enriched population. Percent of CD4+/CXCR4+/CCR5+ T lymphocytes is ~ 15 %.

Table B-1. Intracellular PI levels reported in ng/mL and mM for PBMC treated with 1, 10, or 100 mM IDV or RTV for 3, 18, or 48 hours. Each value was converted from ng/mL as cited in figure 2-3. Ng/mL were converted to grams, then converted to mol using the equation g/MW=mol. Mol were converted to molarity using the equation M=mol/L, where the volume was calculated using the volume of 1 million cells (the number of cells in the sample tested).

Drug	Extracelluar [C] Applied to Cells (μM)	Time Cells Exposed to Drug (Hours)	Intracellular PI Level (ng/mL)	Intracellular PI Level (μM)
IDV	1	3	0.92	3.3
IDV	1	18	0.88	3.1
IDV	1	48	0.61	2.1
IDV	10	3	3.72	13.1
IDV	10	18	3.12	11.1
IDV	10	48	3.27	11.5
IDV	100	3	30.3	106
IDV	100	18	37.0	130
IDV	100	48	36.4	128
RTV	1	3	0.66	2.3
RTV	1	18	1.39	4.9
RTV	1	48	0.32	1.1
RTV	10	3	5.76	20
RTV	10	18	5.36	18.6
RTV	10	48	3.38	11.7
RTV	100	3	242	839.1
RTV	100	18	3088	2059
RTV	100	48	594	1000

Table B-2. Intracellular concentration of RTV in U937 cells treated with 1.0 mM RTV for 18 hours, reported in ng/mL and mM. Each value was converted from ng/mL as cited in figure 2-3. Ng/mL were converted to grams, then converted to mol using the equation g/MW=mol. Mol were converted to molarity using the equation M=mol/L, where the volume was calculated using the volume of 1 million cells (the number of cells in the sample tested).

Sample I.D. **(experiment and replicate #)**	**Intracellular RTV Level** (ng/mL)	**Intracellular RTV Level** (μM)
Expt. #1, Replicate # 1	6.4	6.9
Expt. #1 Replicate # 2	3.2	4.5
Expt. #2, Replicate #1	1.7	2.9
Expt. #2, Replicate #2	2.0	3.6
Expt. #2, Replicate #3	1.6	2.9

Table B-3. Baseline intracellular RTV levels for unstimulated or PHA stimulated PBMC treated with 1 μM RTV for 18 hours at 37oC in 2 % or 10% serum. Each value was converted from ng/mL as cited in figure 2-3. Ng/mL were converted to grams, then converted to mol using the equation g/MW=mol. Mol were converted to molarity using the equation M=mol/L, where the volume was calculated using the volume of 1 million cells (the number of cells in the sample tested).

Donor and Replicate	**PHA Stimulated?**	**[Serum] in Media (%)**	**Intracellular RTV Level (ng/mL)**	**Intracellular RTV Level (μM)**
Donor #1, Replicate #1	YES	2	0.29	4.6
Donor #1, Replicate #2	YES	2	0.32	5
Donor #1, Replicate #3	YES	2	0.28	4.4
Donor #1, Replicate #1	NO	2	0.34	5.4
Donor #1, Replicate #2	NO	2	0.65	10.2
Donor #1, Replicate #3	NO	2	0.26	4.1
Donor #2, Replicate #1	YES	10	0.66	10.4
Donor #2, Replicate #2	YES	10	0.50	7.6
Donor #1, Replicate #1	NO	10	BLQ	BLQ
Donor #1, Replicate #2	NO	10	BLQ	BLQ
Donor #2, Replicate #1	YES	10	BLQ	BLQ
Donor #2, Replicate #2	YES	10	BLQ	BLQ

Table B-4. Intracellular RTV levels in stimulated PBMC in 10 % serum treated with 1.0 mM or 10.0 mM RTV for 18 hours prior to observation of RTV efflux at 4oC or 37oC for 0 (baseline; immediately after 18 hour drug load), 10, 20, 40, or 60 minutes. Each value was converted from ng/mL as cited in figure 2-3. Ng/mL were converted to grams, then converted to mol using the equation g/MW=mol. Mol were converted to molarity using the equation M=mol/L, where the volume was calculated using the volume of 1 million cells (the number of cells in the sample tested).

Donor #	[RTV] Applied to PBMC	Time Cells Allowed to Efflux(Minutes)	Temperature Of Efflux (C)	Intracellular RTV (ng/mL)	Intracellular RTV (μM)
1	1.0	0	4	BLQ	BLQ
1	1.0	10	4	BLQ	BLQ
1	1.0	20	4	BLQ	BLQ
1	1.0	40	4	BLQ	BLQ
1	1.0	60	4	BLQ	BLQ
1	1.0	0	37	BLQ	BLQ
1	1.0	10	37	BLQ	BLQ
1	1.0	20	37	BLQ	BLQ
1	1.0	40	37	BLQ	BLQ
1	1.0	60	37	BLQ	BLQ
1	10.0	0	4	0.98	11.2
1	10.0	10	4	0.98	11.2
1	10.0	20	4	0.94	10.8
1	10.0	40	4	1.5	17.6
1	10.0	60	4	1.96	17.8
1	10.0	0	37	0.34	6.8
1	10.0	10	37	BLQ	BLQ
1	10.0	20	37	BLQ	BLQ
1	10.0	40	37	BLQ	BLQ
1	10.0	60	37	BLQ	BLQ
2	1.0	0	4	0.50	7.6
2	1.0	10	4	0.39	5.9
2	1.0	20	4	0.45	6.6
2	1.0	40	4	0.39	5.9
2	1.0	60	4	0.82	12.4

2	1.0	0	37	0..67	10.4
2	1.0	10	37	BLQ	BLQ
2	1.0	20	37	BLQ	BLQ
2	1.0	40	37	BLQ	BLQ
2	1.0	60	37	BLQ	BLQ
2	10.0	10	37	BLQ	BLQ
2	10.0	20	37	BLQ	BLQ
2	10.0	40	37	BLQ	BLQ
2	10.0	60	37	BLQ	BLQ

Table B-5. Efflux of RTV in stimulated or unstimulated PBMC in 2 % serum treated with 1.0 mM RTV for 18 hours. Efflux of RTV was observed at 4^0C, 20^oC, or 37^oC for 0, 10, 20, 40, 60, or 120 minutes post 18 hour RTV treatment. Each value was converted from ng/mL as cited in figure 2-3.

PHA Stimulated?	Time Cells Allowed to Efflux(Minutes)	Temperature Of Efflux (C)	Intracellular RTV (ng/mL)	Intracellular RTV (μM)
YES	0	4	0.29	4.6
YES	10	4	0.32	5
YES	20	4	0.29	4.6
YES	40	4	BLQ	BLQ
YES	60	4	0.29	4.6
YES	120	4	BLQ	BLQ
YES	0	20	0.32	5
YES	10	20	0.27	4.3
YES	20	20	BLQ	BLQ
YES	40	20	BLQ	BLQ
YES	60	20	BLQ	BLQ
YES	120	20	BLQ	BLQ
YES	0	37	0.28	4.4
YES	10	37	BLQ	BLQ
YES	20	37	BLQ	BLQ
YES	40	37	BLQ	BLQ
YES	60	37	BLQ	BLQ
YES	120	37	BLQ	BLQ
NO	0	4	0.52	8.3
NO	10	4	0.52	8.3
NO	20	4	0.5	7.8
NO	40	4	0.53	8.6
NO	60	4	0.57	9.1
NO	120	4	0.52	8.3
NO	0	20	0.65	10.2
NO	10	20	0.31	5.3
NO	20	20	BLQ	BLQ
NO	40	20	0.40	6.3
NO	60	20	0.44	6.9
NO	120	20	BLQ	BLQ
NO	0	37	0.26	4.1
NO	10	37	BLQ	BLQ
NO	20	37	BLQ	BLQ
NO	40	37	BLQ	BLQ
NO	60	37	BLQ	BLQ
NO	120	37	BLQ	BLQ

Table B-6. Efflux of RTV in stimulated or unstimulated PBMC in 10 % serum treated with 1.0 mM RTV for 18 hours. Efflux of RTV was observed at 40C, 20oC, or 37oC for 0, 10, 20, 40, 60, or 120 minutes post 18 hour RTV treatment. Each value was converted from ng/mL as cited in figure 2-3.

PHA Stimulated?	Time Cells Allowed to Efflux(Minutes)	Temperature Of Efflux (C)	Intracellular RTV (ng/mL)	Intracellular RTV (μM)
YES	0	4	BLQ	BLQ
YES	10	4	BLQ	BLQ
YES	20	4	BLQ	BLQ
YES	40	4	BLQ	BLQ
YES	60	4	BLQ	BLQ
YES	120	4	BLQ	BLQ
YES	0	20	BLQ	BLQ
YES	10	20	BLQ	BLQ
YES	20	20	BLQ	BLQ
YES	40	20	BLQ	BLQ
YES	60	20	BLQ	BLQ
YES	120	20	BLQ	BLQ
YES	0	37	BLQ	BLQ
YES	10	37	BLQ	BLQ
YES	20	37	BLQ	BLQ
YES	40	37	BLQ	BLQ
YES	60	37	BLQ	BLQ
YES	120	37	BLQ	BLQ
NO	0	4	BLQ	BLQ
NO	10	4	BLQ	BLQ
NO	20	4	BLQ	BLQ
NO	40	4	BLQ	BLQ
NO	60	4	BLQ	BLQ
NO	120	4	BLQ	BLQ
NO	0	20	BLQ	BLQ
NO	10	20	BLQ	BLQ
NO	20	20	BLQ	BLQ
NO	40	20	BLQ	BLQ
NO	60	20	BLQ	BLQ
NO	120	20	BLQ	BLQ
NO	0	37	BLQ	BLQ
NO	10	37	BLQ	BLQ
NO	20	37	BLQ	BLQ
NO	40	37	BLQ	BLQ
NO	60	37	BLQ	BLQ
NO	120	37	BLQ	BLQ

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