HIV-1 RESISTANCE TO RILPIVIRINE IN THE CONTEXT OF PRE-EXPOSURE PROPHYLAXIS

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ABSTRACT

To prevent further incidences of human immunodeficiency virus type 1 (HIV-1), pre-exposure prophylaxis (PrEP) is effective at inhibiting infection in high-risk populations; however, PrEP efficacy is correlated with adherence. Currently, the only approved PrEP regimen requires taking daily pills. To improve adherence, rilpivirine (RPV), a non-nucleoside reverse transcriptase inhibitor approved for antiretroviral therapy, has been developed into an injectable long-acting nanoparticle formula (RPV LA). RPV LA provides a depot of drug for sustained release and monthly dosing instead of daily pills. A concern with using approved antiretroviral drugs for both therapy and PrEP is the selection of drug-resistant mutations that may confer cross-resistance to multiple drugs. This issue has public health relevance, as individuals with cross-resistant mutations will have limited therapy options and could transmit drug-resistant virus to others. To study HIV-1 resistance to RPV in the context of PrEP, we treated macaques and humanized mice with RPV LA and characterized selection of drug-resistant virus and the ability to prevent mucosal transmission of drug-resistant HIV-1, respectively. We found that RPV LA monotherapy in macaques selected for transient low-level RPV-resistant mutations. We also found that RPV LA prevented vaginal transmission of HIV-1 with low- but not high-level resistance in a concentration dependent manner. Similar to macaques, we found no evidence of consistent resistance selection in breakthrough infections in mice. Together, these animal models

indicate the risk of resistance selection by RPV LA is low; however, the *in vivo* protective concentration of RPV LA should be better defined before Phase 3 clinical trials to measure efficacy. To counter HIV-1 drug resistance, new antiretroviral compounds must be developed. We studied three experimental RPV analogs and found that one compound selected for a novel combination of two mutations in reverse transcriptase. Together the mutations conferred cross-resistance to all approved NNRTIs; however, hypersusceptibility to several non-NNRTI drugs was observed. This indicates that the analog has a novel interaction with HIV-1 reverse transcriptase that could be exploited to design better antiretroviral compounds.

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PREFACE

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Figure 3 is a composite of figures printed in the following:

1) New England Journal of Medicine, Vol. 328. Pantaleo G, Graziosi C, Fauci, AS. 1993. New concepts in the immunopathogenesis of human immunodeficiency virus infection. Pages 327-335.

2) Cold Spring Harbor Laboratory Press. Fauci AS, Desrosiers RC. 1997. Pathogenesis of HIV and SIV. *In* Coffin JM, Hughes SH, Varmus HE (ed), Retroviruses. Cold Spring Harbor, NY.

3) Cold Spring Harbor Perspectives in Medicine, Vol 2. Swanstrom R, Coffin J. 2012. HIV-1 pathogenesis: the virus. Pages 1-18.

Figure 6 with modifications is reprinted from *Trends in Biotechnology*, Vol. 25. Ambrose Z, KewalRamani VN, Bieniasz PD, Hatziioannou T. 2007. HIV/AIDS: in search of an animal model. Pages 333-337.

1.0 INTRODUCTION

It is estimated that 36.7 million people are infected by human immunodeficiency virus type 1 (HIV-1), with 2.1 million new infections occurring in 2015 (1). Since the discovery of HIV-1, the World Health Organization (WHO) estimates that over 70 million infections and 35 million deaths can be attributed to the virus (2). HIV-1 was discovered and named in the 1980s after healthy young adult men became sick and died from opportunistic infections typically controlled by a normal immune system (3-8). This condition is acquired immunodeficiency syndrome (AIDS) and is defined by a HIV-1 infection plus cluster of differentiation 4 (CD4) positive cell counts of less than 200 cells/μl of blood and/or the presence of opportunistic infections including but not limited to pneumocystis pneumonia, tuberculosis, or Kaposi sarcoma (9). HIV-1 is a human-specific lentivirus that is transmitted via blood-to-blood contact (needle sharing, transfusions, or birth) or exposure to body fluids (genital secretions, breast feeding) with the primary transmission route being sexual intercourse (10). There is no cure for HIV-1, and individuals who become infected require life-long combination antiretroviral therapy (ART) to suppress virus replication, else untreated HIV-1 infection predominantly leads to AIDS.

There is also no approved vaccine to prevent HIV-1 infection because Phase III clinical trials have failed to demonstrate efficacy (11, 12) or showed modest protection (13), the correlates of which are still being explored to improve future vaccination strategies. In the absence of a vaccine, pre-exposure prophylaxis (PrEP) with United States (US) Food and Drug

Administration (FDA)-approved antiretroviral drugs is shown to be effective at preventing HIV-1 infection (Table 1). Currently, the only approved PrEP is daily oral Truvada, a co-formulated pill of the FDA-approved antiretroviral drugs emtricitabine (FTC) and tenofovir (TFV); however, global approval of Truvada is limited. Despite the significant reductions in HIV-1 infections reported in Truvada groups in most PrEP studies, the biggest barrier to efficacy is adherence, as highlighted by lack of protection in study participants not taking their pills in two clinical trials (14, 15).

To improve PrEP adherence, rilpivirine (RPV), an antiretroviral drug approved for ART, was formulated into a long-acting injectable nanoparticle formulation. Long-acting injectable drugs administered by healthcare providers eliminate daily dosing and allow for accurate adherence monitoring. Despite the benefits of injectable PrEP, employing the same antiretroviral drugs (*i.e.*, Truvada or RPV) for both ART and PrEP raises concerns for development or transmission of drug-resistant HIV-1 isolates in newly infected individuals on PrEP. Consequently, drug-resistant HIV-1 can limit future therapy options as mutations may confer cross-resistance to other drugs within the same class (16).

To assess the concern of HIV-1 cross-resistance from PrEP, the theme of this dissertation is to understand the effects of HIV-1 resistance to RPV, either long-acting RPV (RPV LA) or novel RPV analogs. Specifically, *in vivo* models were employed to study the development of drug resistance and inhibition of HIV-1 transmission by RPV LA, while cell cultures were used to determine the genotypic and phenotypic resistance profiles of novel RPV analogs. This research aims to bridge the knowledge gap between *in vitro* and *in vivo* systems to guide future work in HIV-1 prophylaxis.

Table 1. Clinical trials with FDA-approved antiretroviral drugs for HIV-1 PrEP

Trial name	Study population	Study design	Risk reduction in intent-to-treat population	Risk reduction in adherent population	Ref.
iPrEx	MSM, transgender women	daily oral FTC/TFV or placebo	44%	92%	(17)
CAPRISA 004	Women	intercourse-dependent TFV gel or placebo	39%	54%	(18)
FEM-PrEP	Women	daily oral FTC/TFV or placebo	No reduction	Failure due to <40% adherence	(14)
Partners PrEP	Serodiscordant couples	daily oral TFV, FTC/TFV, or placebo	67% (TFV) 75% (FTC/TFV)	86% (TFV) 90% (FTC/TFV)	(19)
TDF2	Serodiscordant couples	daily oral FTC/TFV or placebo	62%	78%	(20)
Bangkok Tenofovir Study	IDU	daily oral TFV or placebo	48.9%	74%	(21)
VOICE	Women	daily oral TFV, FTC/TFV, or placebo, TFV gel or placebo gel	-49% (TFV pill) -4% (FTC/TFV pill) 15% (TFV gel)	Failure due to <30% adherence	(15)
iPERGAY	MSM	intercourse-dependent oral FTC/TFV or placebo	82%	Not reported	(22)
PROUD	MSM	daily oral FTC/TFV immediate or delayed	86% (immediate)	Not Reported	(23)

IDU, injection drug users; MSM, men who have sex with men; FTC, emtricitabine; TFV, tenofovir

1.1 HIV-1, DRUGS, AND RESISTANCE

HIV-1 is a lentivirus of the *Retroviridae* family and has a positive sense, single-stranded ribonucleic acid (RNA) genome. As a retrovirus, HIV-1 enters a cell, reverse transcribes its RNA genome into double-stranded deoxyribonucleic acid (DNA), and then integrates its DNA into the host cell genome to complete the infection cycle. In addition, host cell transcription and translation of the integrated viral DNA is essential for production of new virions. HIV-1 antiretroviral drugs target various stages of the HIV-1 cycle to block infection and production of new virus; however, HIV-1 replication is an error-prone process that can lead to mutations that confer drug resistance, which abrogates ART usefulness.

1.1.1 The HIV-1 replication cycle

The HIV-1 genome consists of nine genes contained within approximately $1x10^4$ bases of RNA that codes for structural, enzymatic, envelope, and accessory proteins essential to infection, replication, and pathogenesis (Figure 1). HIV-1 begins its infection cycle when the virus binds to a receptor on a target cell via viral envelope (Env) proteins. Glycosylated Env proteins (consisting of gp120 and gp41 subunits) on the virion bind to CD4 molecules (24-26), the primary receptor of HIV-1 that can be found on T cells, monocytes, macrophages, and dendritic cells. Interaction of gp120 and CD4 results in the reconfiguration of variable loops in gp120 that also allows binding to target cell co-receptors (27), primarily the chemokine receptors C-C chemokine receptor type 5 (CCR5) or C-X-C chemokine receptor type 4 (CXCR4) (28-30). Conformation changes in gp120 from these host cell interactions exposes gp41, which facilitates fusion of the virus and target cell membranes and entry of the virus core into the target cell (31).

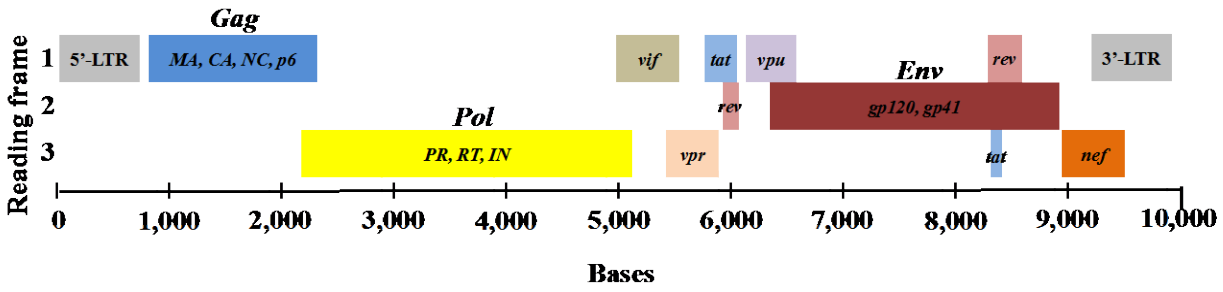

Figure 1. The RNA genome of HIV-1.
HIV-1 utilizes overlapping coding regions in three reading frames to minimize the size of its genome. HIV-1 has three genes, *gag* (blue), *pol* (yellow), and *env* (red) that code for polyproteins. *Gag* encodes the matrix (MA), capsid (CA), nucleocapsid (NC), and p6 proteins that form a protective layer around the genome and accessory proteins and guide the formation of new virions. The *pol* region codes for protease (PR), reverse transcriptase (RT), and integrase (IN), which are responsible for cleavage of polyproteins, transcription of viral RNA into DNA, and integration of viral DNA into the host genome, respectively. *Env* encodes the gp120 and gp41 proteins that create glycosylated heterotrimers necessary for host cell receptor binding and cell entry. Vif, Vpr, Vpu, Tat, Rev, and Nef are accessory proteins that modulate the host cell environment to counter antiviral defenses, activate transcription of viral DNA, and export viral RNA. The reading frames for this figure are based on the HIV-$1_{NL4\text{-}BAL}$ sequence.

The virus core is composed of capsid proteins, encoded in the *gag* gene, that form a protective shell around the viral genome and enzymes (32-35). Upon entry into a host cell, the core will destabilize and shed capsid proteins in a process called uncoating that is important for viral infectivity (36, 37). The extent of uncoating between entry and integration is debated, but core destabilization begins early post-entry (38, 39) and reverse transcription occurs concurrently with uncoating (36, 37).

A hallmark of retroviruses is reverse transcriptase (RT), which is the enzyme responsible for converting the viral single-stranded RNA genome into a double-stranded DNA genome using host cell deoxynucleoside triphosphates (dNTPs) (40, 41). HIV-1 RT is a heterodimeric enzyme made of two subunits, p66 and p51, where p51 is a truncated version of p66 and lacks the ribonuclease H domain (42-46). Synthesis of viral DNA (vDNA) occurs through the enzymatic activity of p66, while p51 provides structural stability (42, 47-49). Thus, RT possesses multiple

enzymatic functions for conversion of single-stranded RNA into single-stranded DNA (RNA-dependent DNA polymerase), conversion of single-stranded DNA into double-stranded DNA (DNA-dependent DNA polymerase), and the removal of RNA bases from the RNA-DNA duplex (ribonuclease H) (42-46). Reverse transcription products are detectable in $CD4^+$ T cells within 4 hours post-infection with peak double-stranded DNA production after 12 hours (50).

At the completion of reverse transcription, the reverse transcription complex will transition into a pre-integration complex (PIC). The PIC is composed of the newly reverse transcribed vDNA genome, capsid, integrase, and host cell nuclear import factors (51-53). After the PIC crosses the nuclear membrane, integrase performs 3' endonuclease processing of the vDNA, bind the vDNA and host cell DNA, nick the host cell DNA, and catalyze the ligation of virus to host DNA (54, 55). Host enzymes repair the DNA gaps to create the provirus (56, 57), which is the stable integrated genome of the HIV-1 virus that is replicated by the host cell. Capsid is also associated with integration as studies show capsid-host factor interactions for targeting the integration of HIV-1 DNA to sites of active replication (58, 59), thus increasing the chances of transcription of HIV-1 genes.

To produce new virions for continuation of the infection cycle, the HIV-1 provirus requires host cell polymerases to produce nascent genomic viral RNA (vRNA) and messenger RNA (mRNA). Host cell transcription factors bind to the 5' long terminal repeat, the *de facto* promoter region of HIV-1, to begin transcription of the provirus (60-63). During early transcription, replication of the provirus is limited to the regulatory proteins Tat and Rev (64, 65). Tat proteins bind to the transactivation-response (TAR) region (66-68) and recruits the positive transcription elongation factor complex to promote quick and efficient transcription of HIV-1 RNA products (69-72). Without the Tat-TAR interaction, HIV-1 transcription stalls early

in the genome (73). HIV-1 transcription involves the production of both spliced and unspliced RNA elements (74). Spliced products include mRNA for Tat, Rev, and Nef, and these transcripts are safely transported out of the nucleus through normal export channels. To avoid degradation by host cell enzymes, unspliced RNA, such as the nascent genome, *gag*, *gag-po*l, *env*, and some accessory proteins, requires the viral protein Rev for nuclear export. Rev binds to the Rev response element found in the *Env* coding region of unspliced vRNA (75-78) and interacts with nuclear pore proteins via a nuclear export signal for safe trafficking of the viral transcripts out of the nucleus and into the cytoplasm (79-81).

After translation of viral mRNA, HIV-1 proteins must coalesce at the cell membrane for proper virion formation and release from the host cell to complete the replication cycle. The Gag polyprotein consists of matrix, capsid, and nucleocapsid domains and recruits Env and binds the genomic vRNA. The Gag-Pol polyprotein contains the same domains along with the protease, RT, and integrase domains. Localization signals in the matrix domain target the transport of these polyproteins to the host cell membrane (82), and domains in nucleocapsid recognize and selectively bind HIV-1 genomic vRNA to ensure packaging of the correct RNA species (83, 84). Host cell granules deliver Env to the cell membrane in a less constitutive manor than Gag and Gag-Pol (85). At the host cell membrane, viral structural proteins multimerize at lipid rafts to begin the budding process (86-91). Immature virions made of viral proteins and host cell membrane moieties are released from the host cell through the action of host endosomal sorting complexes required for transport system (92). Within the nascent virion, viral protease cleaves Gag and Gag-Pol polyproteins to trigger conformation changes necessary for proper core formation and enzyme functions (33, 93-95), thus creating mature, infectious virions and completing the virus replication cycle (Figure 2).

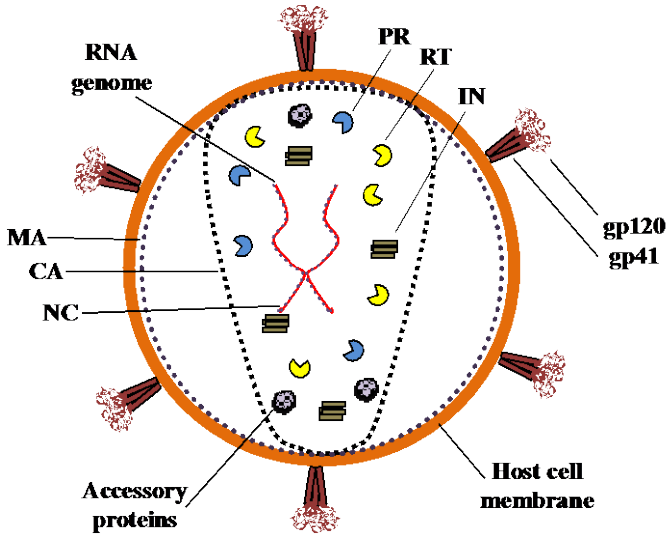

Figure 2. Structure of a mature HIV-1 virion.
Nucleocapsid (NC) forms a protective layer around the genome, while capsid (CA) forms a shell around the genome and viral enzymes (protease (PR), reverse transcriptase (RT), and integrase (IN)) and accessory proteins (Vpr, Vif, Vpu, and Nef) necessary for maturation and infection. Matrix (MA) binds to lipids in the host cell membrane, which become part of the virion membrane as the virus buds. Extracellular envelope proteins (gp120 and gp41) are anchored in the membrane for recognition of target cell receptors and membrane fusion for virus entry.

Throughout the replication cycle, the host cell has a variety of antiviral defenses, but three specialized viral accessory proteins, Vif, Vpu, and Nef, aid in host cell immune evasion and increased pathogenesis. Vif is responsible for binding to apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3 (APOBEC3) enzymes and targeting them to cellular ubiquitin ligases for destruction (96). APOBEC3 proteins are RNA editing enzymes from the APOBEC cytidine deaminase family and can be packaged into virions during nascent virus formation (97). The enzymes deaminate deoxycytidine to create deoxyuridine during first-strand complementary DNA (cDNA) synthesis of reverse transcription, resulting in G-to-A hypermutations that are often lethal to the virus (98, 99). Vpu is a multi-functional protein that 1) binds to CD4 within the endoplasmic reticulum, thus leading to CD4 degradation via proteasomes and allowing proper processing of Env (100-103) and 2) allows virion budding by

antagonizing tetherin, a protein that binds to the virus membrane to prevent release from the host cell (104-107). The third accessory protein, Nef, is also multi-functional. It induces surface CD4 endocytosis by binding to a cytoplasmic domain on CD4 and targeting it for degradation (108, 109). Nef also causes the endocytosis and lysosomal breakdown of MHC class I and class II molecules (110-113), which prevents proper cytotoxic T cell response to infected cells (114) and potentially interferes with antigen presentation and helper T cell stimulation (112), respectively. Additionally, Nef removes SERINC3 and SERINC5 proteins from the cell surface to prevent these molecules from incorporating into virions and inhibiting fusion (115, 116). The loss of any of these accessory proteins diminishes HIV-1 pathogenesis *in vivo* (111, 117-119).

1.1.2 HIV-1 pathogenesis

The primary targets of HIV-1 infection are $CD4^+$ T cells, which have an essential role in host adaptive immunity. There are a variety of $CD4^+$ T cells with distinct functions differentiated by gene expression and cytokine release; however, the overall purpose of these cells is to regulate the adaptive immune response to protect the host from foreign bodies, including viruses. HIV-1 targets these cells through their cell surface expression of CD4 and the chemokine co-receptors, CCR5 and CXCR4, as discussed above. While HIV-1 mainly targets $CD4^+$ T cells, the virus can also productively infect macrophages and dendritic cells (120-126), which also express CD4 (127). Subsequent infection and long-term loss of immune cells serve to deregulate the host immune system and can lead to infection by opportunistic pathogens and the development of AIDS.

Infection of the host begins with transmission of HIV-1. The primary route of infection is through sexual contact, mainly the female and male genital tracts, and rates of transmission vary

depending on exposure route (10). It is estimated that <1% of HIV-1 exposure events in the female or male genital tract lead to an infection, but probability of rectal transmission is estimated as high as 5% due to differences in cell layers and microenvironment (10). Because HIV-1 is a bloodborne pathogen, transmission probabilities are highest for direct blood contact and range from 5-20% or 0.6-95% during pregnancy/birth and direct blood exposure (needles or transfusion), respectively (10). Initial infection is usually attributed to a single founder virus (128-133). However, multiple isolates can infect an individual at one time and may be dependent on virus inoculum, exposure route, and host environment factors (134-139). For mucosal transmission, activated $CCR5^+$ $CD4^+$ T cells are the initial targets of HIV-1 infection (133, 137, 140-145) and are found in high numbers at mucosal exposure sites (146-150), including the gut-associated lymphoid tissue (GALT), which is the largest reservoir of lymphocytes in the human body (151). It is proposed that a specific subset of pro-inflammatory $CD4^+$ cells, called Th17 helper cells, are the first T cells infected by HIV-1 (152-155). In time, HIV-1 evolves the ability to also target CXCR4-expressing T cells, which is linked to increased infectivity and pathogenicity (156-158). Although, macrophages and dendritic cells can be infected by HIV-1, they are not considered major players during transmission and acute infection (143, 145). Newly infected cells migrate to target-rich lymph tissues (*e.g.* lymph nodes (LN)) where the virus can be further disseminated (159), thus exponentially increasing the number of infected cells.

Migration of HIV-1^+ cells from the site of exposure can lead to systemic spread of the infection, setting off the events that lead to AIDS (Figure 3). Plasma viremia reaches a peak approximately three weeks post-infection and is followed by a drop to a viral set-point as the adaptive immune system responds to counter virus replication (160-162). The period of seroconversion and establishment of a viral set-point is known as clinical latency, where an

individual is asymptomatic but the virus is replicating and transmissible to others, and can last for years. The viral set-point is a near steady longitudinal concentration of detectable vRNA copies in the plasma, and higher plasma viremia is associated with clinical progression to AIDS (163-167). However, viremia is not an absolute predictor (168) and progression to AIDS is a long-term multifactorial event involving viremia, immune response, and $CD4^+$ lymphocyte maintenance (163, 165, 166, 169-172). Early signs of HIV-1 infection can be asymptomatic or manifest as a range from general malaise and flu-like symptoms to rashes and unexplained weight loss usually within 4 weeks of infection (9). However, the pathogenic hallmark of HIV-1 infection is the systemic depletion of $CD4^+$ T cells (3-5, 173), particularly in the GALT (174, 175), that leads to disruption of immune homeostasis. This depletion of immune cells is a result of cytotoxicity from HIV-1 production (173), apoptosis or pyroptosis of infected and uninfected neighboring cells (176, 177), and killing of infected cells by cytotoxic immune cells, such as cluster of differentiation 8 (CD8) positive T cells and natural killer (NK) cells (178, 179). Damage to the gut epithelial barrier caused by HIV-1 infection and T cell loss leads to microbial translocation (180-182), and the systemic spread of gut microbe products that further perturb the immune system and cause pathogenicity (183-185). HIV-1 infection is also associated with progressive neurocognitive decline associated with infected macrophages (186-189) and brain atrophy from inflammation and damage to glial cells (190).

HIV-1 is constantly evolving to escape cytotoxic lymphocyte and antibody pressure (191-197), and will eventually cause enough systemic damage to create an immunocompromised environment ideal for invasion by opportunistic pathogens that are normally controlled by a healthy immune system. Such opportunistic infections that led to the discovery of AIDS include *Pneumocystis jiroveci* (formerly *P. carinii*) pneumonia, chronic herpes lesions, and Kaposi's

sarcoma (3-5). Most HIV-1^+ individuals will progress to AIDS in approximately 10 years after infection (198) unless they adhere to an ART regimen that successfully suppresses viremia. ART can significantly delay the onset of AIDS and increases life expectancy of HIV-1^+ persons (199).

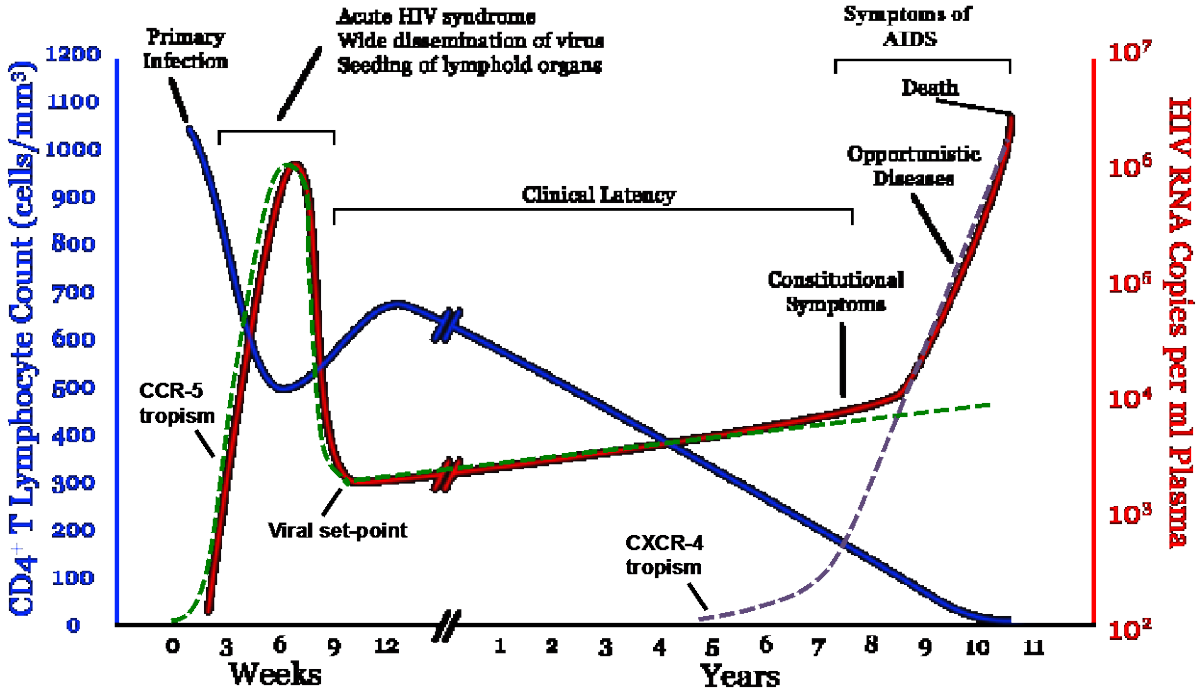

Figure 3. Progression of HIV-1 pathogenesis.
HIV-1 viremia (red) peaks within weeks of infection. During this primary infection, $CCR5^+CD4^+$ cells are preferentially depleted (blue line) but partially rebound as immune functions develop to suppress viremia. The viral set-point and clinical latency occur through a balance of virus replication and immune function. Without ART to suppress viral replication, $CD4^+$ cells will continuously decline due to virus replication until the immune system is compromised to the point that opportunistic infections occur and lead to mortality. Throughout infection, HIV-1 mutates to escape immune pressure and can develop affinity for $CXCR4^+CD4^+$ T cells, which is associated with increased pathogenicity. Figure reproduced and adapted with permission from Pantaleo *et al*. 1993 (200), © Massachusetts Medical Society, and Facui & Desrosiers 1997 and Swanstrom & Coffin 2012 (201, 202), © Cold Spring Harbor Press.

Only one person has been cured of HIV-1. The Berlin patient (named because of the location of successful treatment) was treated with multiple rounds of chemotherapy for acute myeloid leukemia and received an allogeneic stem-cell transplant with donor tissue screened for the HIV-1-resistant delta32 CCR5 allele (203). This allele contains a 32-base pair deletion that

results in a non-functioning CCR5 co-receptor that inhibits cell entry and infection by CCR5-tropic HIV-1 isolates and delays progression to AIDS (204-206). To date, the Berlin patient has undetectable HIV-1 in the absence of ART; however, this success has not been recapitulated in other persons (207) and is not feasible on a global scale. Until a practical cure is discovered, it is crucial to block the HIV-1 infection cycle with ART for HIV-1^{+} individuals and promote effective prophylactic methods to prevent transmission of HIV-1 to uninfected individuals.

1.1.3 HIV-1 antiretroviral therapy

Disruption of any of the steps in the HIV-1 replication cycle can reduce or abolish infection. Because HIV-1 RT, integrase, and protease are not expressed in host cells, these enzymes essential to virus replication are ideal targets for antiretroviral drugs. Currently, there are 25 FDA-approved drugs that target four steps of the HIV-1 infection cycle (Figure 4): entry, reverse transcription, integration, and protease cleavage. Most of these drugs directly bind to their target HIV-1 protein to inhibit proper function.

There are two FDA-approved drugs for inhibiting HIV-1 cell entry, enfuvirtide (T-20) and maraviroc (MVC). T-20 is a 36 amino acid peptide that interacts with the gp41 subunit of HIV-1 Env to prevent fusion of the virus and host cell membranes (208, 209). Alternatively, MVC binds to host cell CCR5 proteins and inhibits gp120 binding; however, the drug is not effective against HIV-1 isolates with CXCR4 tropism (210). MVC is the only FDA-approved HIV-1 drug that targets a non-viral protein. Although both drugs were effective in clinical trials (211, 212); they are not recommended for first-line ART because T-20 requires twice daily injections and is the most expensive therapy, while MVC also requires twice-daily dosing and tropism testing (213).

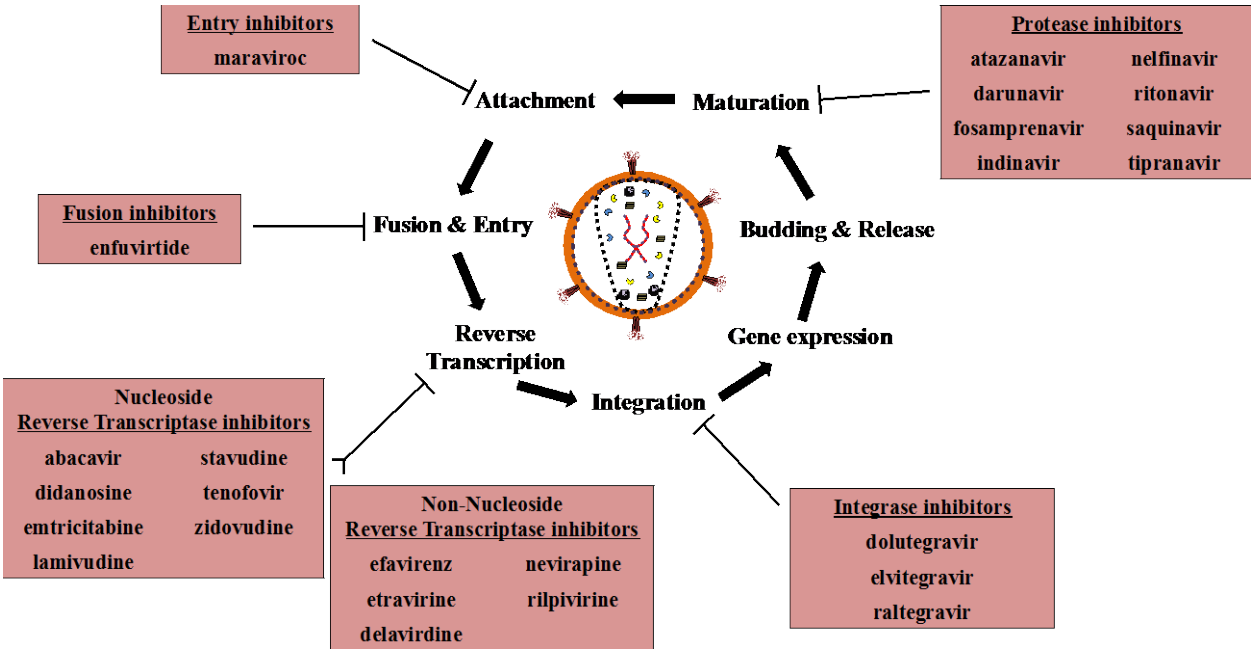

Figure 4. FDA-approved antiretroviral drugs and their targets.
Twenty-five drugs that inhibit five steps of the HIV-1 infection cycle are clinically available for the treatment of HIV-1^+ individuals.

The most commonly prescribed drugs for ART include two classes of RT inhibitors known as nucleoside reverse transcriptase inhibitors (NRTI) and non-nucleoside reverse transcriptase inhibitors (NNRTI). While both drugs target the same step of the HIV-1 infection cycle, their mechanisms of action are distinct. NRTIs were the first drug class approved for HIV-1^+ individuals, and they work by resembling dNTPs but lack a 3' hydroxyl group necessary for DNA elongation. These drugs are phosphorylated within a cell and compete with natural dNTPs for insertion into nascent DNA strands by RT, thus NRTIs are competitive inhibitors and their incorporation results in DNA chain termination and abortive reverse transcription products (214, 215). Conversely, NNRTIs are allosteric inhibitors that hinder RT function by binding directly to RT but not in an active site. These drugs bind with high specificity to a hydrophobic region in HIV-1 RT known as the NNRTI-binding pocket, which is approximately 10 angstroms from the RT polymerase active site (49, 216-218). NNRTIs cause a structural shift in RT that affects the

alignment of the primer terminus and the polymerase active site, thus inhibiting the chemical step of nucleotide transfer (219-221). A dual combination of NRTIs (abacavir and lamivudine or FTC and TFV) forms the backbone for all first-line and most alternative recommended ART regimens in the US (213); however, the NNRTIs efavirenz (EFV) and RPV are reserved for alternative first-line options because of central nervous system side effects (EFV), reduced efficacy based on dose (RPV), single mutation resistance profiles (both), and increasing prevalence of resistance mutations within HIV-1^+ populations (both) (213).

Integrase inhibitors are the newest class of drugs approved by the FDA for ART. There are three clinically available integrase inhibitors that bind directly to the enzyme to prevent strand transfer of the viral genome to the host DNA (222, 223). An advantage of integrase inhibitors over RT inhibitors is the larger window of time for them to be transported into a cell to be effective, as integration does not begin until approximately 8 hours post-entry (50). However, these drugs are only functional after integrase complexes with HIV-1 DNA (222). Due to the potency and tolerability of integrase inhibitors, all three are recommended as first-line HIV-1 therapy in combination with NRTIs in the US (213).

The fourth and largest class of antiretroviral drugs is the protease inhibitors. These drugs bind to the catalytic domain of protease and prevent Gag and Gag-Pol polyprotein cleavage that is necessary for viral maturation and infectivity (94, 95). Most protease inhibitors have peptide-like centers that allow direct interaction with the catalytic aspartic acid residues of HIV-1 protease (224, 225). However, this class of antiretrovirals is only effective in cells where HIV-1 has integrated and begun to produce new virions. Of the eight approved protease inhibitors, darunavir in combination with two NRTIs is recommend for first-line therapy, and atazanavir and lopinavir with two NRTIs are recommended for alternative starting regimens; however,

protease inhibitors require a booster drug, ritonavir, and tend to be more toxic than integrase and RT inhibitors (213).

In 2015, WHO revised global guidelines on ART and now recommends that all HIV-1^+ individuals should receive ART regardless of $CD4^+$ cell counts (226). Previous guidelines recommended ART for all HIV-1^+ children regardless of CD4 T cell count and adults with <350 $CD4^+$ cells/ml or AIDS-related conditions because of improved development and less clinical events in children (227-230) and reduced mortality in adults (231). However, the new recommendations are based on more recent data that show reduction in transmission and better clinical outcomes if ART is started immediately rather than until AIDS occurs (232-236). These studies comparing early versus deferred ART showed significant reductions in HIV-1-related clinical events (232-236), a decrease in mortality (233, 234), and a 96% drop in transmission within serodiscordant couples (232, 237) in cohorts that received immediate ART. Thus, immediate therapy leads to earlier suppression of HIV-1 viremia and better conservation of the immune system, which results in reductions in transmission and adverse clinical events.

1.1.4 HIV-1 resistance selection

The HIV-1 replication cycle is a dynamic process that involves virus proteins as well as dependence on host cell factors that can each exert distinctive rates of mutagenesis. The high mutation and turnover rates of HIV-1 virions lead to genetic variation that can create drug-resistant mutations (DRMs) within the viral quasispecies, the heterogeneous virus population detectable in each infected individual. DRMs can lead to HIV-1 replication in the presence of ART, thus causing therapy failure and increasing the probability of drug-resistant virus transmission (238).

To complete a replication cycle, HIV-1 requires RT to transcribe viral genomic RNA to DNA, and host RNA polymerase II (Pol II) to synthesize proviral DNA into viral genomic and mRNA. The function of these two enzymes can introduce mutations into the HIV-1 genome. The mutation rate of RT in various biochemical and cell culture assays is estimated to range from 10^{-4} to 10^{-6} bases per replication cycle (239-245). Mutations caused by RT arise from mismatched base substitutions, base deletions or insertions, and frameshift from enzyme slippage in homopolymeric regions (241, 242, 246, 247). HIV-1 RT lacks the proofreading capability to fix errors generated during reverse transcription (239, 240) and is considered the driving force behind HIV-1 mutagenesis (243, 246). The exact contribution of human RNA Pol II to HIV-1 mutation rates is unknown, but RNA Pol II has proofreading capabilities suggesting its role in generating mutations would be significantly lower than RT (246, 248).

Because an infectious HIV-1 virion contains two separate strands of RNA, there is potential for RT to produce mutations through genomic hybridization. Recombination of HIV-1 genomes occurs via strand displacement during reverse transcription when RT switches from one RNA strand to another (249, 250). Should a cell be infected with multiple subtypes of HIV-1 (discussed in the proceeding section), it is possible for nascent virions to package heterologous genomes that could recombine to create a novel infectious isolate referred to as a unique or circulating recombinant form (CRF) (251). High rates of recombination are described in cell lines (252, 253); however, *in vitro* recombination in primary T cells and macrophages is reported to average 1.5 and 6.2 events per 1,000 bases (245). The higher recombination rate in macrophages is attributed to lower dNTPs pools (254), which prompts increased DNA synthesis pausing, strand transfer efficiency, and template switching (255-257). Thus, HIV-1 RT influences HIV-1 evolution through infidelity and recombination.

As mentioned before, the APOBEC3 family of proteins can also contribute to HIV-1 mutagenesis; however, controversy remains as to the contribution of APOBEC3-mediated viral mutagenesis to HIV-1 pathogenesis in humans (258, 259). If unimpeded, the direct contribution of APOBEC3 proteins to drug resistance is likely low due to the high mutation rates and tendency to generate replication incompetent virus (98, 99, 260). APOBEC3 proteins have been estimated to contribute 10- to 100-fold more mutations than RT (258), but the frequency of sublethal mutations is >6-log less than the mutation rate of RT (259). APOBEC3 activity can be reduced by interaction with Vif, thus decreasing the number of APOBEC3-driven mutations and replication-incompetent genomes (261). Hence, APOBEC3 proteins can influence HIV-1 evolution. Interestingly, APOBEC3G activity was shown to prime the quasispecies for easier selection of the M184I RT resistance mutation through recombination (262); although, APOBEC3 hypermutations are not associated with increases in recombination rates (259).

The most significant factor associated with HIV-1 evolution and DRM development is the high turnover rate of the virus. The production of new virions has been estimated to be approximately 10^{10} virions per day with half the plasma viral population turning over nearly every six hours (263). At its lowest estimated fidelity, RT could generate one base change per viral genome per replication cycle. Combined with the production rate of new virions, each base of the HIV-1 genome could change >10,000 times a day (264). Because of rapid mutation and high turnover rates, the virus quasispecies within an individual is estimated to be 3,000 generations removed from its founding sequence after a decade (264). Thus, the longer an individual has unchecked virus replication, the larger the genetic variation and the greater the likelihood of developing DRMs, particularly in replication competent proviruses.

1.1.5 HIV-1 resistance and antiretroviral therapy

HIV-1 can develop DRMs that reduce susceptibility to all approved antiretroviral drugs (16). Because antiretroviral drugs within the same class tend to bind to the same regions of their respective targets, DRMs reduce drug binding and can lead to cross-resistance of several or all drugs of the same class. Such cross-resistant patterns lead to limited therapy options in HIV-1^+ individuals who acquire or develop these mutations through transmission or evolution, respectively. For example, single amino acid changes in the NNRTI-binding pocket such as K103N or G190S confer high-level resistance to both EFV and nevirapine (NVP) with no change in susceptibility to RPV or etravirine (ETR) (265). However, four mutations in RT, L100I, K101P, G190E, and M230L are considered to have clinically relevant resistance to all four FDA-approved NNRTIs (16, 266). Similarly, mutations at positions T69 and Q151 in RT, or at Q148 in integrase reduce the utility of all NRTIs and integrase inhibitors, respectively (16, 266). MVC is the exception in that HIV-1 can develop mutations to allow binding in the presence of the drug or evolve the preference for an alternative co-receptor (*e.g.* CXCR4) (267).

Despite access to anti-HIV-1 drugs since the late 1980s, treatment failure due to replication of drug resistant virus was noted in individuals taking single drug regimens, or monotherapy (268-270). To prevent resistance selection, the current practice of combination ART (formerly referred to as HAART: Highly Active Antiretroviral Therapy) replaced monotherapy in 1996 as clinical data in individuals on a multi-drug regimen showed better viremia suppression (271-274), immune cell recovery (271-273, 275), and reduced resistance selection (272, 273, 276). Therefore, it is imperative that HIV-1^+ individuals adhere to a lifetime regimen of three to four drugs from at least two antiretroviral drug classes for successful viremia suppression.

Because of its high mutation rate, decades of replication before the advent of ART, and global compartmentalization, HIV-1 group M has evolved into nine distinct subtypes and dozens of CRFs (277). CRFs are a result of infection by more than one HIV-1 subtype and genetic recombination that creates a virus with a mixed subtype genome (278-280). Nearly 50% of the global HIV-1^{+} population is infected with subtype C virus, predominantly in southern Africa and Middle East nations (281); however, the majority of antiretroviral drug development uses subtype B HIV-1, which is most prevalent in the US and Western Europe (281). This means the differences between subtypes may affect the global utility of antiretroviral drugs.

Genetic drift and recombination can lead to polymorphisms, which are different amino acids that are prevalent at the same position within a population, and can influence drug resistance. Between subtypes, 53% of the entire protease and 48% of the first half of the RT coding region are polymorphic in untreated individuals (282). Although most DRMs are similar across subtypes (282), polymorphisms at positions associated with HIV-1 drug resistance could lead to suboptimal therapy in non-B dominant areas. For example, the E138A RT mutation is associated with RPV resistance (16, 265, 283) and is significantly more prevalent in untreated individuals infected with subtype C than subtype B virus (284). Consequently, RPV may not be as effective in areas where subtype C HIV-1 is prevalent, as using the drug could lead to treatment failure in individuals with the mutation.

Despite the benefits of ART and increasing improvements in global ART coverage (1), there is a global increase in drug-resistant HIV-1 prevalence (285). Subsequently, geographic regions with higher treatment rates could see a shift towards drug-resistant polymorphisms if the drugs are not utilized properly. ART could lead to increased DRM prevalence by mutations from suboptimal therapy pressure and drug-resistant HIV-1 transmission to uninfected persons. Thus,

it is imperative to continually develop novel, potent, and safe drugs and therapy combinations that can counter HIV-1 with currently known DRMs and any unique resistant mutations that arise to future drugs.

1.1.6 Rilpivirine

RPV is a diarylpyrimidine small molecule that belongs to the NNRTI class of antiretroviral drugs (Figure 5) and was approved by the FDA for ART in 2011. RPV is the most potent FDA-approved NNRTI to date with *in vitro* effective concentrations to prevent 50% infection (EC_{50}) ranging from 0.06-1.01 nM against primary and laboratory HIV-1 isolates and displays no loss of susceptibility against resistance mutations associated with the first generation NNRTIs, EFV and NVP (265, 286, 287). RPV potency is accredited to amino linker bonds, which provide flexibility to reposition the drug within the NNRTI-binding pocket, and the cyanovinyl group that binds a hydrophobic tunnel between the NNRTI-binding pocket and the dNTP-binding cleft (286, 288, 289). The cyanovinyl group is unique to RPV and not found on the other clinically relevant diarylpyrimidines, ETR and dapivirine (DAP) (286).

Results from clinical trials showed RPV to be non-inferior to EFV in reducing plasma viremia in HIV-1-infected participants on combination ART (290-293). However, RPV was tolerated better, caused significantly less side effects, and predominantly selected different resistance mutations than EFV (292-295). The selection of distinct resistance mutations without cross-resistance to EFV suggests that HIV-1^+ individuals would be able to continue with NNRTIs as part of their ART regimen should DRMs develop to either drug. In the US, RPV in combination with Truvada is recommended as an alternative first-line ART regimen because of increased failure in individuals with plasma viremia >$1x10^5$ RNA copies/ml (213, 292).

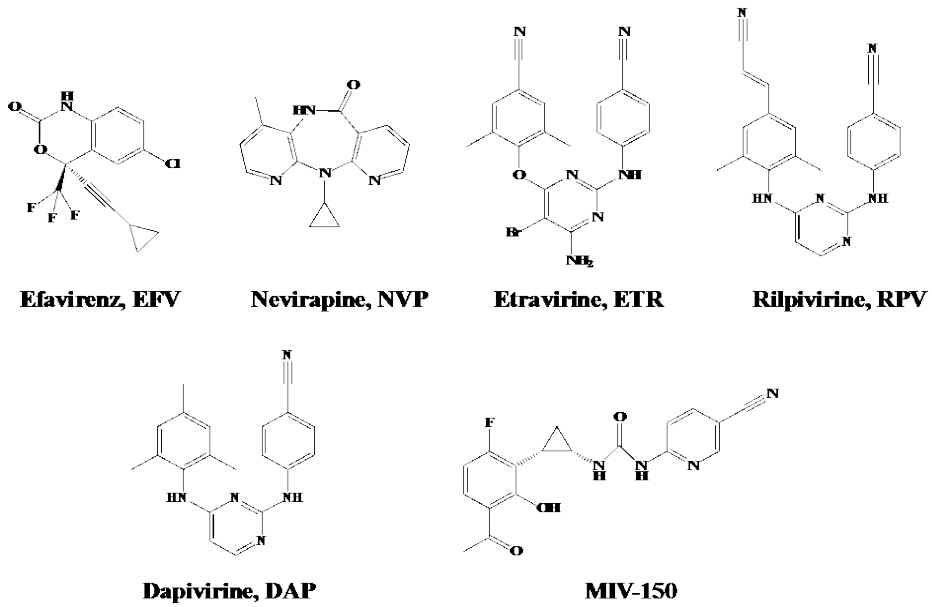

Figure 5. Chemical structures of clinically relevant NNRTIs.
EFV, NVP, ETR, and RPV are all FDA-approved NNRTIs for antiretroviral therapy. DAP and MIV-150 are not currently approved for clinical use. However, DAP formulated in an intravaginal ring is shown to protect against vaginal HIV-1 transmission. MIV-150 as part of a multi-functional vaginal gel for PrEP is in safety trials.

1.1.7 HIV-1 resistance to RPV

As previously discussed, HIV-1 can develop mutations that reduce the potency of antiretroviral drugs, some of which can confer cross-resistance to multiple compounds. Because all NNRTIs target the same hydrophobic region of RT (49, 216, 217, 286), DRMs within and around the NNRTI-binding pocket reduce the ability of the drug class to inhibit HIV-1 replication through a reduction in enzyme-drug interactions (296-302). The NNRTI-binding pocket is considered to encompass positions L100, K101, K103, V106, T107, V108, V179, Y181, Y188, V189, G190, F227, W229, L234, P236, and Y318 of p66 and E138 of p51 (49, 216), and clinically relevant mutations to FDA-approved NNRTIs include V90I, A98G, L100I, K101E/H/P, K103N/S, V106A/I/M, V108I, E138A/G/K/Q/R, V179D/F/L/T, Y181C/I/V, Y188C/H/L, G190A/S,

H221Y, P225H, F227C, M230I/L (16). For example, the more rigid first generation NNRTIs EFV and NVP predominantly have reduced ability to occupy the NNRTI-binding pocket in the presence of DRMs, such as K103N and Y181C. The K103N mutation in RT results in the formation of hydrogen bonds between N103 and Y188 and water molecules that 1) essentially block drug access to the NNRTI-binding pocket (296) or 2) result in less drastic conformation changes in drug-bound RT (297). In Y181C RT, NVP loses ring-stacking interactions in the mutant enzyme, which decreases binding affinity (298). However, the different side chain interactions and structural flexibility of RPV (and diarylpyrimidines) allows the drug to reposition within mutant RT and maintain inhibitory activity against viruses with DRMs that confer resistance to EFV and NVP (265, 286, 288).

Although most NNRTI-associated resistance mutations require only one base change (303), RPV is considered to have a higher genetic barrier to resistance than first generation NNRTIs. The genetic barrier to resistance is defined as the number of mutations needed to confer resistance to a drug, and the decrease in drug susceptibility from a single amino acid mutation is less pronounced for RPV than EFV or NVP (265). For example, K103N is the dominant mutation selected in adults failing EFV and NVP therapy (304, 305), but is not selected by RPV and does not confer resistance (265, 306). RPV predominantly selects for E138K (265, 295, 307). In an *in vitro* drug susceptibility assay, HIV-1 with K103N showed a >30-fold decrease in susceptibility to EFV and NVP, while E138K and many other drug-resistant HIV-1 clones showed an approximate 3-fold or less decrease in susceptibility to RPV (265). Theoretically, high enough concentrations of RPV should still suppress virus with DRMs conferring low-level resistance as opposed to EFV and NVP, which tend to select for resistance mutations that display substantial changes in susceptibility. Known single amino acid mutations that confer >10-fold

reduction in susceptibility to RPV are K101P, Y181I, and Y181V, which all require two base changes; else, HIV-1 requires more than one known drug-resistant mutation for high-level resistance to RPV (265). This resistance phenomenon is a hallmark of diarylpyrimidines and was also noted with ETR (308).

In cell culture and RPV clinical trials, E138K, K101E, and Y181C were the most commonly selected DRMs (265, 283, 295, 306). The linker groups of RPV form H-bonds with K101 of the p66 subunit and E138 of the p51 subunit of RT, allowing the drug to bind to the virus enzyme and hinder reverse transcription (288, 289). Mutations at these positions break a salt bridge that links the RT subunits (300) and creates a wider gap in the NNRTI-binding pocket that reduces RPV binding stability (301, 302). Mutations at the Y181 position result in a loss of stacking interaction between the tyrosine group of RT and the dimethylphenyl ring of RPV, again reducing binding of RPV (288). However, the flexibility of RPV allows the molecule to shift within RT to create or maintain other interactions with the enzyme, which accounts for the low-level of resistance conferred by many mutations in the NNRTI-binding pocket region. In the case of Y181C, the ring moiety of RPV can alternatively interact with the tyrosine residue at position 183 to maintain its ability to bind and inhibit RT function, though reduced (288). The RT-binding locations and shifting rings of RPV are critical for potency and why first-generation NNRTI-associated DRMs confer little to no reduction in susceptibility to the drug. The resistance mechanisms conferred by other RPV-associated mutations have not yet been explored.

As of this writing, half of the clinically relevant RPV-associated mutations are associated with DRM transmission but only K101E and Y181C are found in >5% of the treatment-experienced HIV-1^{+} population, while all RPV-associated DRMs are reported in <0.5% of treatment-naïve populations (266, 309). The global distribution of RPV DRMs is varied, with

Europe and Latin America having the highest prevalence of RPV resistance populations at 3.2% and 3.6%, respectively (310). However, drug-resistant polymorphisms at the E138 position are found in 6.1%, 5.1%, and 3.3% of individuals infected with subtypes C, F, and A, respectively (310). While E138 mutations currently have an overall low global prevalence, Y181C is the second most prevalent NNRTI-resistant mutation worldwide with 17.5% prevalence in ART-experience populations (285). Combined, these data indicate that RPV must be used with caution in certain geographic regions or in patients infected with particular HIV-1 subtypes.

If RPV use becomes more common, it is likely the prevalence of associated resistance mutations will also increase and potentially reduce the efficacy of RPV to be effective against HIV-1. Despite a restricted ART recommendation, the favorable potency, safety, and tolerability profile of RPV is why it was chosen for development as a long-acting injectable PrEP agent (311, 312). The use of FDA-approved drugs for both ART and PrEP brings into question the effects of PrEP on resistance selection and DRM transmission. However, a reduction in use of NNRTIs for ART, as recommended by US guidelines (213), could eventually reduce prevalence and incidence of NNRTI-resistant infections, thus alleviating the concern with drug-resistant HIV-1 transmission. This would allow RPV to remain a viable PrEP option should it be shown effective in animal models and clinical trials.

1.2 PRE-EXPOSURE PROPHYLAXIS

PrEP is the use of antiretroviral drugs by uninfected individuals to prevent HIV-1 transmission by providing localized or systemic drug concentrations prior to virus exposure. Effective drug concentrations at sites of exposure, particularly mucosal tissues, should entirely block infection

or avert spread of a founder population of HIV-1-infected cells. In clinical trials, either daily oral TFV or Truvada was found to significantly prevent HIV-1 infection in high-risk populations including men and transgender women who have sex with men (MSM) (17, 23), serodiscordant couples (19), young heterosexual adults in an area of high HIV-1 prevalence (20), and injection drug users (21) (Table 1). Similarly, significant protection has been shown with intercourse-dependent Truvada (22) and 1% TFV topical gel (18) (Table 1). At the time of this writing, only daily oral Truvada for PrEP is available in high-risk populations, but global availability is limited. Despite the success of Truvada PrEP in clinical trials, adherence, resistance selection, resistance transmission, and long-term use of drugs by HIV-1$^-$ individuals are areas of concern.

1.2.1 HIV-1 PrEP

There are 25 approved antiretroviral drugs and countless analogs and experimental compounds that could be chosen for PrEP. The cost and time to properly evaluate a fraction of these candidates would require vast resources and would significantly delay control of the HIV-1 pandemic. Therefore, drugs already approved for clinical use are more likely to be accepted as PrEP because of known safety, tolerability, resistance, and efficacy profiles, which entails years of thorough study and large-scale clinical trials before approval. For example, Truvada was an excellent candidate for PrEP because of good bioavailability of the active metabolites, a well-established safety profile, and lack of interaction with non-HIV-1 therapies relevant to other conditions/diseases (17). The NRTIs in Truvada, FTC and TFV, mimic separate dNTPs (cytidine and adenosine, respectively) and select for distinct resistance mutations (313, 314), indicating a therapeutic effect could be maintained should an individual acquire a single DRM while on PrEP. Of importance, animal models showed successful prevention of HIV-1 infection by

Truvada PrEP (315, 316), providing *in vivo* evidence of PrEP utility prior to initiating clinical trials.

To test the efficacy of daily oral Truvada as PrEP, thousands of individuals from several nations participated in multiple randomized, blinded, and placebo-controlled clinical trials. The rates of protection within the total populations were 44%, 62%, and 75% in MSM, sexually active adults, and serodiscordant couples, respectively (17, 19, 20). When the efficacy data for these trials were adjusted to analyze participants with detectable drug concentrations, HIV-1 prevention was >75% for adherent individuals. Even if drug was detected in treated individuals who became infected, the concentrations were significantly lower than those of matched uninfected persons (17, 20). Comparison of TFV concentrations and protection in Truvada clinical trials indicates that plasma TFV concentrations of ~50 ng/ml estimates a 50% risk reduction in HIV-1 infection (317) and specifies an obtainable, protective concentration in individuals. The success of Truvada PrEP is a much-needed boon for HIV-1 prevention, and different compounds and delivery modalities are being explored to increase PrEP options.

Similar to ART, development of DRMs is a concern for PrEP efficiency. Individuals who are HIV-1^{+} at initiation or become infected while on PrEP will essentially be treated with mono- or dual therapy, which could select for drug resistance similar to what occurred in the 1980s and early 1990s prior to the initiation of pre-combination ART. PrEP-turned-therapy and DRM selection raises the issue of cross-resistance and limited ART options; however, resistance selection in individuals adherent to Truvada was low. Follow-up analyses of seroconverters in the TDF2 and iPrEx trials revealed no selection of DRMs if the individuals were infected after initiating PrEP (318, 319); however, 4.8% and 3.3% of infected participants in the Partners PrEP study developed a resistant mutation as a result of Truvada or TFV PrEP, respectively (320).

These results indicate that selection of drug resistance during Truvada PrEP is low if one is infected after initiating PrEP. Yet, the resistance outcome was different, though rare, for persons who received PrEP when already infected with HIV-1. One of three participants retrospectively found to be infected at the start of the TDF2 study developed mutations resistant to both drugs (318), while 25% of HIV-1^+ participants with undetected infection given PrEP in the Partners trial developed DRMs (320). Two participants in the iPrEx trial were retrospectively discovered to be HIV-1^+ prior to enrollment, and the data indicate that FTC exposure selected M184I/V mutations quickly (319). Although the data sets are small, it appears the risk of developing resistance as a result of PrEP is unlikely if the infection occurs after starting PrEP. However, the individuals in these trials were closely monitored. In a clinical setting, the amount of time before detection of HIV-1 infections in PrEP users may allow more time for selection of DRMs.

While efficacy and low resistance selection for daily oral Truvada PrEP has been observed, adherence is the largest barrier to PrEP effectiveness and should be addressed to reduce the global burden of HIV-1. Daily oral PrEP was ineffective in two studies, the FEM-PrEP and VOICE trials, as no difference in infection rates was exhibited between treatment and placebo groups (14, 15). The entire FEM-PrEP and the single drug arms of VOICE were halted early because of such discouraging results. Despite greater than 85% adherence based on product return and self-reporting, <40% of participants were actually found to be adherent based on plasma drug concentrations in both trials. Follow-up interviews revealed that poor adherence was associated with low risk perception (321) and fear of side effects, real or imagined (322). Although adherence measured by plasma drug concentrations never matched rates of self-reporting or product count monitoring in any Truvada clinical trial (14, 15, 17, 19, 20), adherence in the successful trials was considerably higher. In the iPrEx trial, age and higher

education levels were positively correlated with adherence (323). Similarly, increased age was also positively correlated with adherence in the Bangkok Tenofovir (21) and VOICE (15) trials. These results suggest maturity and experience as key factors for adherence; however, with increased implementation of PrEP strategies and proper education and outreach, it is expected that adherence will increase for future product use (324).

To address daily adherence issues with PrEP, alternative regimens such as intermittent oral pills, topical gels, and intravaginal rings (IVRs) have been investigated, but not yet approved. In the iPERGAY trial, intercourse-driven oral Truvada use rather than daily pills led to an 82% reduction in HIV-1 infections in a MSM population (22). These results indicate that intermittent oral dosing may alleviate adherence problems, as users would take PrEP only during times of sexual activity, thus assuaging fears of potential long-term side effects from daily use.

Microbicide gels provide localized concentrations of anti-HIV-1 compounds and were originally proposed as intercourse-driven PrEP controlled by women (325). The CAPRISA 004 trial explored the use of an intercourse-dependent 1% TFV vaginal gel as PrEP and reported a 39% reduction in HIV-1 infections in the overall treated group, but a 54% reduction in women who were adherent to the product (18). Conversely, no difference in HIV-1 transmission between gel or placebo groups was reported in the VOICE study because of poor adherence (15). Several other microbicide trials explored the use of carrageenan-based gels, Carraguard and PRO2000 (326-328); the surfactant C31G (329); and the acidic Buffergel (328) and all of them failed to show efficacy or were associated with increased infection risk (330, 331). Microbicide gels are not limited to vaginal formulations, as rectal gels with TFV or an experimental NNRTI are shown to be safe and tolerable in both men and women (332-334).

Another female-driven PrEP strategy is the IVR, which provides a solid substrate for localized long-term drug release with intermittent replacement and have been prescribed for contraception and hormone replacement for decades (335). An IVR containing the NNRTI DAP showed similar results to 1% TFV topical gel, leading to 37% and 56% lower incidences of HIV-1 transmission among the total and adherent treated populations, respectively (336). A second DAP IVR clinical trial showed 31% lower incidence of HIV-1 in the DAP group; however, there was no correlation between adherence and increased protection (337). Women would apply the IVR and replace as necessary to maintain vaginal drug concentrations, thus eliminating concerns of missed daily doses. Despite promising results, intermittent pills, microbicide gels, and IVR strategies are still user-driven and cannot guarantee adherence.

New long-acting injectable formulas of antiretroviral drugs such as the NNRTI RPV or the integrase inhibitor cabotegravir (CAB) have been developed for PrEP and have favorable safety and dosing profiles (338-340). Long-acting injectable formulations are designed specifically for intermittent use and provide sustained systemic release of drugs instead of localized concentrations, like topical gels or IVRs. This strategy has been successful in fields such as cancer treatment, contraception, hormone replacement, and psychiatry (341-344). Long-acting injectable PrEP benefits from the elimination of user responsibility for daily or event-driven dosing and administration and adherence monitoring by healthcare providers. Opinions on taking injectable drugs as PrEP are positive in target populations, such as MSM and high-risk women (345, 346); however, long-acting PrEP has not been tested in efficacy trials at the time of this writing.

1.2.2 RPV LA as PrEP

Because of its aforementioned potency and safety profiles, RPV is the most appropriate clinically approved NNRTI for PrEP (312). RPV has been formulated as a long-acting injectable designed to provide a depot of drug that is released systemically over time for monthly dosing and better adherence. RPV LA is prepared by wet-milling irradiated RPV with a surfactant (polxamer 338) in an aqueous environment to obtain a nanoparticle suspension with an average particle size of 200 nm (311, 312, 347). Chelating and isotonifying agents and a phosphate buffer are then added to preserve the product for long-term refrigerated storage (312).

Phase I clinical trials showed that RPV LA is safe and tolerable with reported low-grade adverse events such as rash, stiffness, and injection site pain and swelling (338, 340, 348). In one clinical trial, pharmacokinetic (PK) analysis of plasma RPV in women administered a single 300, 600, or 1,200 mg intramuscular (IM) injection displayed a dose-dependent effect on plasma and vaginal tissue drug disposition (338). Maximal plasma drug concentrations in women averaged 160 ng/ml at 6 days post-injection in the 1,200 mg group and were sustained to 83 ng/ml by 28 days post-injection for a calculated half-life of 38 days (338). The ratio of drug concentrations in cervicovaginal lavages and vaginal tissues versus plasma at 28 days post-injection averaged 1.0 and 0.8, respectively (338). The decay of lavage and plasma drug concentrations mirrored each other; however, vaginal tissue-to-plasma ratios increased as tissue drug concentrations remained steady (300 mg and 600 mg) or increased (1,200 mg) indicating that RPV may concentrate in vaginal tissue, while it is metabolized in plasma (338). In the same study, a population of men was given a 600 mg IM injection of RV LA. Average plasma RPV concentrations were higher in men at all time points than women given the same dose, but displayed a nine day shorter half-life indicating gender difference in metabolism (338). Male rectal tissue-to-plasma ratios of RPV

averaged 0.9 at 2 weeks post-injection (338), suggesting that plasma RPV concentrations could be used as a surrogate measurement for rectal drug concentrations. Data from all participants administered the 600 mg dose suggests that RPV penetrates rectal tissue better than vaginal tissue as concentrations in rectal tissue were 3-fold higher one week post-injection (338), a characteristic of RPV LA that has been confirmed in explant models (348, 349). Although a minimal *in vivo* RPV protective concentration for RPV LA has not been defined to date, the authors reference a protein-adjusted effective concentration to prevent 90% ($PAEC_{90}$) value of 12.1 ng/ml and propose this as a baseline measurement as a protective threshold (338). Overall, the authors demonstrated systemic distribution of RPV and deposition in sites relevant to HIV-1 exposure at concentrations above the theoretical protective concentration.

A second RPV LA clinical trial corroborates the dose-dependent plasma drug concentrations reported by Jackson *et al*. (338) and outlined a potential dosing strategy of priming with a 1,200 mg IM injection followed by monthly 600 mg injections (340). This dosing regimen was tolerable, maintained drug concentrations above the proposed protective 12.1 ng/ml $PAEC_{90}$ value, and revealed a consistent peak and trough of plasma RPV concentrations maintained within a 50-80 ng/ml range (340), the minimum effective range reported in oral RPV trials (338). Current methods of measuring RPV account for total drug concentrations and not free/active drug. Like most NNRTIs, RPV is highly protein bound (265, 350) and reported drug concentrations are not an accurate representation of available active drug. While the results of both studies support testing of a monthly 1,200 mg RPV LA dosing strategy for PrEP, it is crucial to determine *in vivo* protective concentrations of RPV and the feasibility of providing these thresholds in humans.

To understand RPV penetration and protection in mucosal tissues, human tissue explant models were used to determine protective ranges of RPV *ex vivo*. Ectocervical and colon explants revealed that RPV concentrates more readily in colonic versus female genital tract tissue (348, 349). In one study, tissues were incubated in media containing RPV before exposure to HIV-1, and the colonic explants exhibited significantly higher RPV absorption than ectocervical explants, which absorbed <10% of the input drug (349). The effective concentration to prevent 90% infection (EC_{90}) for RPV in the ectocervix and colon explants was approximately 99 and 16 ng/ml, respectively, thus displaying a 6-fold difference in concentrations needed for protection between the tissue compartments (349). A second study used explants taken from RPV LA-treated individuals and found that RPV concentrates in rectal tissue up to 2.5-fold more than matched vaginal and cervical tissues (348). However, this study reported an EC_{90} value of 91 ng/ml in rectal tissues sampled 14 days post-injection and no protective correlate in cervical or vaginal tissues, which suggests that a 1,200 mg dose of RPV LA may not be effective (348). If these explant results are applied to reported human RPV tissue concentrations (338), RPV LA should provide protection from rectal exposure, suggesting that RPV LA may not be an ideal PrEP candidate for women at risk of HIV-1 infection via vaginal exposure. The explant models further emphasize the need for careful characterization of the *in vivo* protective concentrations of RPV LA, which appear to be unique to specific tissue compartments.

Along with mucosal tissue penetration and protective concentration concerns with RPV LA, cold storage, shipping chains, and a long detectable pharmacologic tail have brought into question the practicality of the PrEP agent. A single 1,200 mg dose of RPV LA provides concentrations of drug that are still detectable nearly 170 days post-dosing; however, after 40 days post-dosing, the drug concentrations remain just above the aforementioned theoretical

protective concentration (338, 348). This long detectable tail of RPV is a problem because low drug concentrations could be ideal for resistance selection should a user stop receiving RPV LA and then become infected with HIV-1. Such resistance selection did occur in one individual that received a single 300 mg dose of RPV LA in the SSAT 040 trial and became infected during the tail phase when RPV concentrations hovered near the 12.1 ng/ml EC_{90} for over 200 days (351). Using a sensitive sequencing technique, the RPV-associated mutation K101E was detected in approximately 20% of the viral population 70 days after infection (351). Although this is a singular event, it shows that resistance selection can occur during RPV LA monotherapy.

Currently, a phase II clinical trial is underway to explore the safety and tolerability of repeated 1,200 mg IM doses of RPV LA administered to low-risk HIV-1^- females every 8 weeks for 52 weeks (https://clinicaltrials.gov; NCT02165202). Given the scope of the trial, the supply chain issues may be addressed; however, questions with protective thresholds and waning RPV concentrations should be answered before RPV LA is moved into large scale Phase III trials.

1.2.3 Transmission of drug resistance

While resistance selection during PrEP appears to be limited based on results from clinical trials, the question of the ability of PrEP to prevent transmission of drug-resistant HIV-1 still remains. The global rollout of ART is necessary for the control of HIV-1 viremia and reduction of incidences (232, 237); however, increased ART coverage positively correlates with prevalence of drug resistance (352, 353). According to a meta-analysis, overall drug-resistant HIV-1 prevalence has increased in Sub-Saharan Africa, Latin America, North America, and upper income Asian regions since the scale-up of ART; however, prevalence of NNRTI-associated DRMs and odds of drug-resistant virus transmission is increasing in every global region (285).

Because drug-resistant HIV-1 can be transmitted, global surveillance of specific mutations that are associated with virologic failure was initiated (309, 354). The four criteria for inclusion as a surveillance resistance mutation are: 1) the mutation must be associated with drug resistance and recognized on multiple expert lists of resistance mutations; 2) the mutation should be non-polymorphic, nor occur at highly polymorphic sites; 3) the mutation must show resistance across the most common subtypes of HIV-1; and 4) the list should be simple and exclude very rare mutations (354). Currently there are 34 NRTI, 19 NNRTI, 40 protease, and 17 integrase-associated surveillance mutations (http://hivdb.stanford.edu/pages/surveillance.html). A concern is that PrEP will not inhibit transmission of drug-resistant HIV-1; therefore, the continued scale-up of ART combined with PrEP availability could push the global HIV-1 population towards drug-resistant genotypes.

Because of cross-resistance between currently approved drugs, transmission of resistant virus could limit therapy options of newly infected individuals and reduce PrEP options for serodiscordant partners. Of the oral Truvada clinical trials, only one participant from the Partners PrEP study was reported to have acquired a transmitted drug-resistant infection, which contained NRTI- and NNRTI-associated mutations (320); however, this participant was considered non-adherent to the regimen, so PrEP failure may not have been to blame. Thus far, two case studies have reported transmission of multi-drug-resistant HIV-1 in individuals adherent to Truvada PrEP (355, 356). In both cases, FTC-associated and NNRTI-associated DRMs were detected, including E138Q (355) and Y181C (356). Both E138Q and Y181C confer resistance all NNRTIs (265), thus limiting the utility of an NNRTI-containing ART regimen in these individuals. No resistance was reported in the intermittent Truvada (22) or 1% TFV gel (18) trials. In the ASPIRE study, DRM transmission occurred in clinical trial participants using DAP IVRs.

Similar prevalence of DRMs were reported between newly infected individuals in the placebo and treatment DAP IVR groups (336), and population sequencing results suggest that these seroconverters acquired NNRTI-associated DRMs through transmission (357). In contrast, the development of a DAP-associated DRM, E138A, was more frequent in the treated group versus the placebo in the Ring Study (337).

Transmission of drug-resistant HIV-1 is detected at rates between 2.8-11.5% and is associated with ART use and geographic region (285). NRTI-associated mutations account for the majority of transmissions, and the likelihood of acquiring resistant virus to multiple drug classes decreases by at least 4-fold per additional class (285), suggesting the case studies described above may be rare events and difficult to account for in the design of a clinical trial. Studying PrEP efficacy against drug-resistant HIV-1 is hindered by not knowing the genotype or the amount of virus in which clinical trial participants are exposed. The design of the Partners-PrEP trial, with matched serodiscordant couples, is the only ethical way to test resistance transmission in humans; however, it was not discussed if any of the HIV-1^{+} partners in the trial had a drug-resistant genotype (19). Because transmitted resistance is infrequent and not ethical to purposefully test in humans, animal models have been developed to test the efficacy of PrEP against drug-resistant virus transmission.

1.2.4 Animal models for HIV-1 infection

Cell culture-based studies cannot accurately reflect the complexities of pathogen infection in a host. For example, a novel therapeutic compound that targets a pathogen may be highly effective *in vitro* but host factors such as bioavailability, metabolism, and tissue penetration may affect clinical efficacy. Thus, robust *in vivo* models should be utilized to study such intricate

interactions. To truly understand host and pathogen interactions, it is ideal to study routes of infection, disease pathogenesis, and therapeutic strategies in the host species. However, HIV-1 is a human-specific pathogen with no cure, thus it would be unethical to intentionally infect people to study the virus and its associated disease and treatment. The use of animal models in the HIV-1 field links *in vitro* discoveries to *in vivo* studies and mitigates the high costs associated with long-term human trials that require hundreds to thousands of participants. Animal studies permit manipulation of experimental variables such as genetic backgrounds of the animals, virus challenge stocks, time and route of infection, duration and adherence of treatment, invasive sampling, and other factors that cannot be controlled or sanctioned in human trials. Animals allow researchers to logically and ethically model specific scenarios for hypothesis-driven investigation and provide *in vivo* proof-of-concept data that can guide clinical trial designs or predict study outcomes. Currently, the two most common animals used for HIV-1 modeling are macaques and humanized mice.

Phylogenetic evidence strongly suggests that HIV-1 evolved from multiple simian immunodeficiency virus (SIV) cross-species transmissions from African non-human primates (NHP) (358-360), and has been circulating in the human population since the early 1900s (361). However, the SIV crossover responsible for the majority of the HIV-1 pandemic originates from chimpanzees (360), the family of animals most genetically related to humans (362). However, HIV-1 does not readily infect NHPs nor recapitulate AIDS disease progression (363). SIV does not progress to AIDS in its natural African primate hosts, and pathogenesis is abrogated by sustained regulation of immune functions and control of chronic immune activation, which are deregulated hallmarks of infection in non-natural primate species or HIV-1 in humans (364, 365). However, a serendipitous discovery in the mid-1980s revealed that Asian rhesus macaques

could be infected with SIV to recreate a disease pattern that resembles AIDS in humans (366, 367). Historically, the rhesus macaque (*Macaca mulatta*), an Old World monkey endogenous to central Asia, is the most widely used animal for HIV-1 model research, but other species of Asian macaques, including pigtailed (*Macaca nemestrina*) and cynomolgus (*Macaca fascicularis*), are also utilized (368).

SIV or simian-human immunodeficiency virus (SHIV) in macaques offers the advantage of mirroring HIV-1 infection in humans through similar target cells, immune response, and pathogenesis. SHIVs are chimera viruses that contain coding regions from HIV-1 in the SIV genome to model the effects of HIV-1-specific treatments or prevention strategies in NHPs (369-372). Infection of macaques with SIV recapitulates the pathogenesis hallmarks of HIV-1 infection including $CD4^+$ T cell depletion (373), microbial translocation (182), and AIDS (366, 367). In addition, macaques are outbred, which allows for modeling of infection within hosts of varying genetic backgrounds. There are many well-characterized SIV and SHIV strains that can be used for NHP infection (374); however, consideration is warranted when choosing a NHP species and challenge virus because of differences in viremia levels, disease progression and pathogenesis, characterization and diversity of protective MHC alleles and innate immune factors, efficacy of therapy, and physiologic cycles (368, 374, 375). For example, female rhesus and pigtailed macaques both experience menstrual cycles, but the former has seasonal cycles while the latter has monthly cycles more comparable to human women (376, 377). Greater thinning of the vaginal epithelium in pigtailed macaques during the luteal phase makes them more susceptible to vaginal infections than rhesus macaques (378).

Despite the knowledge gained from modeling SIV and SHIV infections in NHPs, neither virus is HIV-1, and results from macaque models using either virus should be validated in the

context of HIV-1 infection in humans. HIV-1 cannot be used to recapitulate AIDS in macaques because of NHP restriction factors. Macaque variations of tripartite-motif-containing protein 5α, APOBEC3, tetherin, and SAM domain- and HD-domain-containing protein 1 inhibit HIV-1 replication in macaques due to the lack of the necessary HIV-1 factors evolved by SIV to counter these immune mechanisms (379-383). Also, while most anti-HIV-1 drugs will work against SIV (384-386), differences between SIV and HIV-1 RT (387, 388) prevent NNRTIs from working against SIV. For *in vivo* studies of NNRTIs, a chimeric RT-SHIV virus is used for infection of NHPs (369, 370, 389). Aside from physiological limitations, macaques studies can be limited by availability, facility space, and high costs associated with purchase and maintenance. Hence, an ideal macaque model would be much less expensive and permit the use of HIV-1.

A second animal model for studying HIV-1 infection is the humanized mouse. Humanized mice are generated by transplant and engraftment of human cells and/or tissue into an immunodeficient mouse strain for recapitulation of a human immune system in a small animal (390). Procedures for creating humanized mice can be as simple as an intravenous (IV) injection of human peripheral blood lymphocytes or as complicated as invasive surgery for the transplantation of human tissues (391). Humanized mice provide the key advantage of an *in vivo* system in which actual HIV-1 and human cell response can be studied. Mice are also readily available to order from vendors or can be bred quickly, are significantly less expensive than NHPs, are easier to handle, and require less space and maintenance, thus allowing for larger cohorts.

There are several different methods and genetic backgrounds that can be utilized to generate humanized mice (390). Severe combined immunodeficiency (*scid*) mice transplanted with fetal thymus and LN tissue and liver cells (392) were first shown to be susceptible to HIV-1

infection in the late 1980s (393). However, less technically challenging humanized mouse models include *Scid* mice only injected with human peripheral blood leukocytes (394, 395) or cluster of differentiation 34 (CD34) positive hematopoietic stem cells (396). *Scid* mice have a mutation in the protein kinase, DNA activated, catalytic polypeptide (*Prkdc*^{scid}) gene that causes defects in DNA repair and inhibits the generation of B and T cells (397, 398), which allows engraftment of human cells with limited host immune response to the non-mouse cells. However, these *scid* models display leaky B and T cell development (399) and NK cell activity that rejects human cell engraftment (400). Recombination-activating gene (*Rag1/2*) deficient mice are deficient in adaptive immune cell development and eliminate B and T cell leakiness, but still have active NK cells (401, 402). The crossbreeding of *scid* or *Rag1/2* with non-obese diabetic (NOD) mice generated an immunodeficient mouse line that lacks both adaptive immune and NK cell activity (403-405). However, the addition of mutations in the interleukin-2 receptor gamma chain (*IL2rg*) to the *scid*/NOD or *Rag1/2*/NOD lines further impairs B, T, and NK cell development and function (406-408) and provides the highest level of human cell engraftment compared to the aforementioned strains (409-412). These different genetic lines (and others) can be used to for HIV-1 infection and treatment studies; however, the model principally used for HIV-1 PrEP research is the bone marrow, liver, and thymus (BLT) mouse. To generate BLT mice, immunodeficient animals are irradiated and transplanted with human fetal liver and thymus tissue (413-416). Post-tissue transplantation, the mice are injected IV with autologous human CD34^{+} hematopoietic stem cells isolated from the fetal liver and allowed to rest for 8-12 weeks until human immune cells reconstitute the recipient. The genetic backgrounds recommended for BLT surgery are NOD-*Prkdc*^{scid}*IL2rg*^{Tm1Wjl} (NSG), NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Sug}, or C.129(Cg)-*Rag2*^{tm1Fwa9}*Il2rg*^{tm1Cgn} (391). The presence of the human

thymus provides stem cells with an environment for education and HLA-restriction of human origin. These mice also develop human immune cell reconstitution at mucosal tissues (315, 417-419), a feature not seen in the aforementioned humanized mouse models (368). However, BLT mice do have limitations such as a shortened life span due to graft versus host disease (420), poor lymphoid structure development (421, 422), limited B cell maturation (423), and the necessity for human cytokines for improved human myeloid and NK cell function (424). Unlike NHPs, large volumes of blood and survival tissue biopsies are not practical in mice for longitudinal survival studies. Assays that require plasma (*e.g.* measurement of plasma viremia) have reduced sensitivity in mouse samples due to the trade-off between sampling frequency and safe blood draw volumes. In addition, tissue samples require euthanizing animals. Finally, the immunodeficient genetic background of these animals requires stringent aseptic handling and housing to prevent secondary infections that could cause confounding morbidity or mortality.

Although not perfect because of physiological and anatomical differences, animal models serve as pre-clinical systems to evaluate ART and PrEP regimens before more expensive human testing. Therefore, there will always be a need to use animals to safely transition from drug discovery to clinical trials and provide proof-of-concept for antiretroviral agents and prophylactic regimens.

1.2.4.1 Macaque models for PrEP

Macaques have been utilized to test PrEP strategies for over 20 years. Studies with macaques have mirrored the success of human PrEP trials with daily oral Truvada (17), intermittent oral Truvada (22), and microbicide gels (18). In animal models, there are two virus challenge paradigms applied: a single high-dose or repeated low-dose model. The reasoning for the former model is that if an agent can protect from a single high-dose virus challenge, usually beyond

physiologic norms, then success should logically extend to lower more realistic inoculums. This paradigm allows for quicker, less complicated studies. The latter model is employed to mimic physiological HIV-1 inoculums to which humans would be repeatedly exposed (425, 426). There have been dozens of studies published using both challenge paradigms to study PrEP efficacy with experimental and approved compounds in macaques. As previously discussed, approved antiretroviral drugs are more likely to also be accepted for prophylaxis, and Table 2 summarizes the design and outcome of studies that employed macaque models to assess approved antiretroviral drugs as PrEP.

PrEP intervention with approved NRTIs was shown to be viable as early as 1992. In a small study, two infant macaques were protected from SIV infection when administered oral AZT before and after IV challenge (427); however, this success was not repeated in a follow-up study with newborn macaques given a different PrEP dose and virus challenge (428). Because of frequent dosing and side effects, AZT is not an ideal candidate for PrEP, but these studies provided proof-of-concept for pre-exposure intervention with small molecules to inhibit HIV-1 infection. TFV, considered a more tolerable and potent drug than AZT, proved to be a more suitable PrEP candidate as daily subcutaneous (SC) injections protected 100% of juvenile macaques from a high-dose SIV challenge (453). Subsequent studies with low and high doses of oral TFV administered prior to and after oral SIV challenge prevented infection in nearly 100% of newborn macaques in an effort to mimic mother-to-child transmission during breast-feeding (429, 431). Conversely, regular oral TFV dosing exhibited little to partial protection from multiple oral SIV challenges in infant macaques (432, 434); however, it was concluded that protection was dose-dependent implying that a fixed dose was not adequate to provide protective drug concentrations as the animals matured (434). Discrepancies between these studies can be

Table 2. Macaque studies using FDA-approved antiretroviral drugs as PrEP to prevent mucosal transmission of SIV or SHIV

NHP species	PrEP (dose)	Dosing Strategy	Virus	Challenge (route)	Outcome	Ref.
Rhesus (infant)	oral AZT (50 mg/kg)	2 h pre-challenge and every 8 hours for 6-10 weeks	SIV_{mac}	single, low dose (IV)	2/2 protected	(427)
Rhesus (newborn)	oral AZT (25 mg/kg)	2 h pre-challenge and every 8 hours for 15 months	SIV_{mac}	single, high dose (IV)	0/3 protected, significantly better clinical outcomes	(428)
Rhesus (newborn)	SC TFV (30 mg/kg)	4 h pre- and 20 h post-challenge	SIV_{mac251}	single, high dose (oral)	4/4 protected	(429)
Rhesus (newborn)	SC TFV (30 mg/kg)	1 d pre-challenge and continued daily for 4 weeks	K65R SIV_{mac055}	2 high doses 24 h apart (oral)	2/5 protected, delayed disease course	(430)
Rhesus (newborn)	SC TFV (4 mg/kg)	4 h pre- and 20 h post-challenge	SIV_{mac251}	single, high dose (oral)	3/4 protected	(431)
Rhesus (infant)	oral TFV (~0.01-0.02 mg/kg)	Full dose once daily 1.5 h pre-challenge or 2 half doses daily 1.5 h pre-challenge and 2.5 h post-challenge	SIV_{mac251}	repeated, low dose (oral)	1/4 protected (1 full dose) 2/4 protected (2 half doses)	(432)

Table 2 continued

Rhesus	oral TFV (22 mg/kg)	1) 2 h pre-challenge then daily for 36 weeks 2) 2 h pre-challenge then weekly for 36 weeks	$SHIV_{162p3}$	repeated, low dose (rectal)	1/4 protected (daily) 0/4 protected (weekly) median infection time: 1.5 weeks (untreated), 6.0 weeks (weekly), 7.0 weeks (daily)	(433)
Rhesus (infant)	oral TFV (10 mg/kg)	1) 6 d pre- and 1 d post-challenge 2) 2 d pre- and 2 weeks post-challenge	SIV_{mac251}	1) 5x daily, low dose (oral) 2) 5x daily, low dose (oral)	1) 5/6 protected 2) 5/6 protected follow-up challenges resulted in 60% infection of those initially protected	(434)
Rhesus	TFV gel (1%)	1) 15 min pre-challenge 2) 120 min pre-challenge	$SIV_{mac251/32H}$	single, high dose (rectal)	1) 4/6 protected 2) 2/3 protected	(435)
Rhesus	1) SC FTC (20 mg/kg) 2) oral FTC/TFV (20 & 22 mg/kg) 3) SC FTC/TFV (20 & 22 mg/kg) 4) SC FTC/TFV (20 & 22 mg/kg)	1-3) daily7-9 d pre-challenge then daily for 18 weeks 4) 2 h pre-challenge and 24 h post-challenge	$SHIV_{162p3}$	repeated, low dose (rectal)	1) 2/6 protected 2) 4/6 protected 3) 6/6 protected 4) 6/6 protected significant delay in infection in PrEP vs. untreated groups	(316)

Table 2 continued

Rhesus	1-3) oral FTC/TFV (20 & 22 mg/kg)	1) 22 h pre-challenge and 2 h post-challenge 2) 3 d pre- and 2 h post-challenge 3) 7 d pre- and 2 h post-challenge 4) 2 h pre- and 26 h post-challenge	$SHIV_{162p3}$	repeated, low dose (rectal)	1) 5/6 protected 2) 5/6 protected 3) 4/6 protected 4) 3/6 protected significant delay in infection in PrEP groups vs. untreated	(436)
Rhesus	1) MVC gel (0.25 mM) 2) MVC gel (0.5 mM) 3) MVC gel (2 mM) 4-5) MVC gel (6 mM)	1-4) 30 min pre-challenge 5) 0.5-12 h pre-challenge	$SHIV_{162p3}$	single, high dose (vaginal)	1) 2/4 protected 2) 3/4 protected 3) 3/4 protected 4) 6/7 protected 5) 50% efficacy reduction at 4 h pre-challenge	(437)
Rhesus	oral FTC/TFV (20 & 22 mg/kg)	3 d pre-challenge and 2 h post-challenge	M184V $SHIV_{162p3}$	repeated, low dose (rectal)	5/5 protected	(438)
Rhesus	MVC gel (3.3%)	30 min pre-challenge	$SHIV_{162p3}$	single, high dose (vaginal)	4/4 protected	(439)
Rhesus	oral FTC/TFV (20 & 22 mg/kg)	3 d pre-challenge and 2 h post-challenge	K65R $SHIV_{162p3}$	repeated, low dose (rectal)	5/6 protection, 10x the K65R inoculum needed to be as infective as WT virus	(440)

Table 2 continued

Rhesus	oral MVC (44 mg/kg)	1 d pre-challenge and 2 h post-challenge	$SHIV_{162p3}$	repeated, low dose (rectal)	1/6 protected	(441)
Rhesus	1) TFV gel (1%) 2) MVC gel (1%) 3) TFV/MVC gel	30 min pre-challenge	$SHIV_{162p3}$	repeated low-dose (rectal)	1) 4/6 protected 2) 4/6 protected 3) 4/6 protected	(442)
Pig-tailed	1) TFV gel (1%) 2) TFV/FTC gel (1 and 5%)	1-2) 30 min pre-challenge	$SHIV_{162p3}$	repeated, low dose (vaginal)	1) 6/6 protected 2) 6/6 protected	(443)
Pig-tailed	TFV gel (1%)	3 days pre-challenge	$SHIV_{162p3}$	repeated, low dose (vaginal)	4/6 protected	(444)
Pig-tailed	oral FTC/TFV (20 & 22 mg/kg)	24 h pre-challenge and 2 h post-challenge	$SHIV_{162p3}$	repeated, low dose (vaginal)	6/6 protected	(445)
Pig-tailed	IVR TFV (10% by weight)	1 week pre-challenge, replaced every 4 weeks	$SHIV_{162p3}$	repeated, low dose (vaginal)	6/6 protected	(446)
Pig-tailed (DMPA-treated)	oral FTC/TFV (20/22 mg/kg)	24 h pre-challenge and 2 h post-challenge	$SHIV_{162p3}$	repeated, low dose (vaginal)	6/6 protected	(447)
Pig-tailed	TFV gel (1%)	30 min before challenge	K65R $SHIV_{162p3}$	repeated, low dose (vaginal)	5/6 protected	(448)
Pig-tailed	IVR TFV (10% by weight)	1 week pre-challenge, replaced every 4 weeks in presence of DMPA	$SHIV_{162p3}$	repeated, low dose (vaginal)	5/6 protected 1 infected after 8 challenges	(449)

Table 2 continued

Pig-tailed	1) oral FTC/TFV (20/22 mg/kg) 2) TFV IVR (60 mg) 3) TFV gel (1%)	1) 24 h pre-challenge and 2 h post-challenge 2) 1 week pre-challenge 3) 30 min pre-challenge	1) $SHIV_{162p3}$ 2) $SHIV_{162p3}$ 3) K65R $SHIV_{162p3}$	1-2) repeated, low dose (vaginal) 3) repeated, high dose (vaginal)	1) 6/6 protected 2) 3/6 protected 3) 5/6 protected	(450)
Pig-tailed (STI^+)	oral FTC/TFV (20/22 mg/kg)	24 h pre-challenge and 2 h post-challenge	$SHIV_{162p3}$	repeated, low dose (vaginal)	4/6 protected	(451)
Pig-tailed	IVR FTC/TFV (68 & 65 mg)	1 week pre-challenge	$SHIV_{162p3}$	repeated, low dose (vaginal)	6/6 protected (bi-weekly IVR changes)	(452)
Cynomolgus (juvenile)	1) SC TFV (20 mg/kg) 2) SC TFV (30 mg/kg)	2 d pre-challenge and continued daily for 4 weeks	SIV_{mne}	single, high dose (IV)	1) 5/5 protected 2) 10/10 protected	(453)

AZT, zidovudine; DMPA, depot medroxyprogesterone; FTC, emtricitabine; IV, intravenous; MVC, maraviroc; SC, subcutaneous; STI, sexually transmitted infection; TFV, tenofovir

attributed to design dissimilarities such as drug dose, route, and animal age. Yet, the conclusions emulate lessons from the Partners PrEP and TDF2 trials where infection in participants with detectable TFV was associated with lower plasma drug concentrations (19, 20), suggesting a specific drug concentration that needs to be achieved and maintained for protection. Of note, it was shown that active TFV concentrations greater than the *in vitro* effective concentration to prevent 95% of infection in vaginal lymphocytes is correlated with protection in macaques from vaginal transmission (444), providing a reference for mucosal protection in females for future studies.

Although newborn and infant macaque studies provided pre-clinical data on the benefits of PrEP, the majority of HIV-1 incidences in humans are a result of sexual transmission among adults (454). Two studies directly modeled the ability of TFV-based PrEP to prevent rectal infection in adult macaques to support the results of Truvada clinical trials. Once-daily or weekly oral TFV administered two hours prior to repeated rectal challenges with low-dose SHIV delayed infection by 5-6 challenges in treated animals; however, the overall infection rate was similar between the groups (433). Daily oral Truvada also reduced infection 7.8-fold in another study and was shown to be superior to FTC alone (316). These two studies provided proof-of-concept that daily oral PrEP could provide protection or delay infection in groups that are at risk of HIV-1 transmission via rectal exposure, which was the concern addressed in the iPrEx trial (17). A similar pre- and post-exposure dosing design successfully modeled intermittent Truvada PrEP to inhibit SHIV infection, too. When given 22 hours, 3 days, or 7 days prior to challenge followed by a dose two hours post-challenge, intermittent Truvada was as protective as daily oral dosing (436). These results mirror the outcome of the iPERGAY trial, which showed an 82% reduction in rectal HIV-1 infections in MSM that used intercourse-dependent oral Truvada (22).

Although not explored in humans yet, intermittent oral Truvada was also shown to provide full protection against repeated vaginal challenges in macaques (445).

Alternative NRTI delivery strategies, such as IVRs containing TFV have been tested as PrEP in macaques as well. The TFV IVR provided consistently high drug concentrations in vaginal fluid and local tissues in macaques (446, 449) and were significantly higher than those reported to be protective in human women using a 1% TFV gel (18). The TFV IVR provided significant protection or delay in infection from repeated low-dose vaginal SHIV challenges in cycling and hormone-treated pigtailed macaques (446, 449). Interestingly, one of the studies showed infection inhibition in an IVR preparation also containing a contraceptive hormone (449), which provides *in vivo* proof-of-concept for a multipurpose IVR. Such a multifunctional product could empower women in areas such as Africa where the HIV-1 prevalence is higher in young females than males (455). To expand the utility of IVRs, work has begun on a product that provides contraception and the ability to block HIV-1 and sexually transmitted viruses (456).

Like NRTIs, NNRTIs have also been modeled in macaques as PrEP. However, NNRTIs are not effective against SIV unlike other antiretroviral drug classes (384-386). NNRTI resistance in SIV is due to a 60% homology difference between the RT coding regions of SIV and HIV-1 (387) and NNRTI-resistance mutations that occur naturally in SIV RT (388). To address this issue, a chimeric virus that replaces the SIV RT sequence with the RT coding region of HIV-1_{HXB2} (457) in the SIV genome was created and named RT-SHIV (Figure 6). Two versions of RT-SHIV were developed using either SIV_{mac239} (369) or SIV_{mne} (370) as backbones for use in rhesus or pigtailed macaques, respectively. *In vitro* drug susceptibility of RT-SHIV is similar to that of HIV-1 (370, 386, 458-460). Infection of macaques with RT-SHIV leads to viremia and disease progression equivalent to SIV (369, 461, 462) and responds to NNRTI

therapy with viral suppression and evolution of resistance mutations documented in human treatment failures (369, 389, 463-468). Despite the availability of RT-SHIV, only a few experimental NNRTIs have been investigated as PrEP in macaques.

Two phenylethylthioureathiazole compounds, MIV-150 and MIV-160 (469), were each explored as microbicide gels and IVRs using the RT-SHIV challenge model. MIV-150 was originally co-formulated with Carraguard as a topical gel and shown to be highly effective at preventing vaginal and rectal infection when applied 30 minutes before challenge in animals with or without a herpes simplex virus type 2 (HSV-2) co-infection (470-472). Zinc acetate was then added to the gel because of its potential to block HSV-2, and the new formula prevented 100% of vaginal RT-SHIV infections when applied within 4-24 hours of repeated challenges (473, 474). However, the gel was only 50% effective in preventing rectal infection within 8 hours of use (474), indicating a limited window of efficacy. Unfortunately, the gel formulation was not successful at preventing HSV-2 infection (475). MIV-150 was also tested in an IVR delivery system and provided significant protection when placed two weeks or 24 hours before challenge and left in place for two weeks post-challenge (476). Removal of the ring soon after challenge abrogated the inhibitory effect demonstrating that prolonged use of the product is necessary to prevent infection. The related compound MIV-160 was 100% effective when delivered by an IVR but showed no protection combined in a Carraguard/zinc acetate gel and has not moved into clinical testing (477). Although only the gel formulation of MIV-150 is currently in safety trials (https://clinicaltrials.gov; NCT02033109), macaque models demonstrate the potential of MIV-150 to be administered in two separate delivery systems to increase user options for PrEP while eliminating less effective candidates (MIV-160).

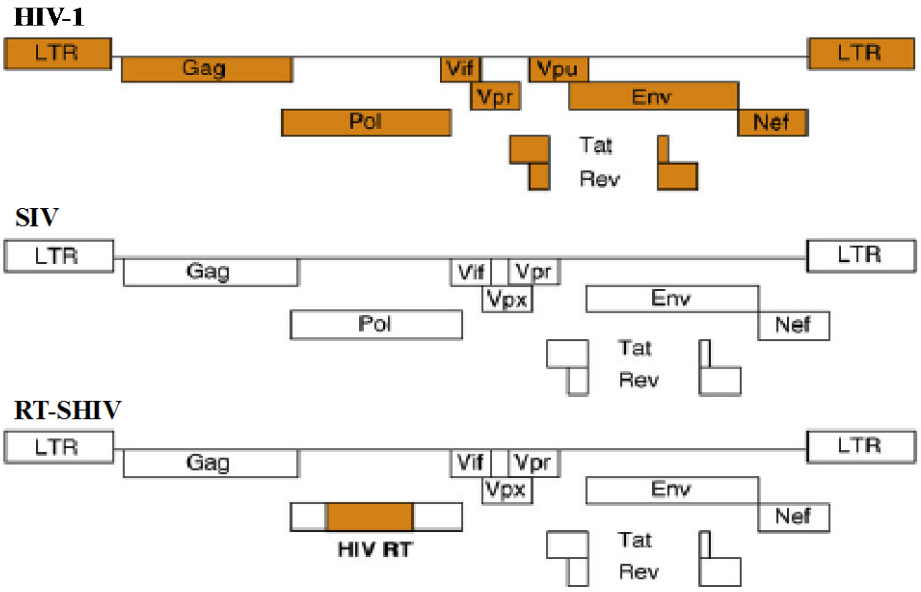

Figure 6. Comparison of the HIV-1, SIV, and RT-SHIV genomes.
The RT coding region of SIV is replaced with the RT coding region of HIV-1 to create a virus that is infectious in macaques and susceptible to NNRTIs. Figure reproduced and adapted with permission from Ambrose et *al*. 2007 (478), © Elvesier.

MC1220, a member of the 3,4-dihydro-2-alkoxy-6-benzyl-4-oxopyrimidines family of NNRTIs (479), was tested as a topical vaginal microbicide and IVR. A 0.5% or 1.5% gel formulation provided 50% protection in rhesus macaques when challenged with a high dose of RT-SHIV 30 minutes after gel application (480). This result was replicated in a follow-up study, but efficacy was nonexistent in a 0.1% gel formulation (481), suggesting a protective threshold beginning at a 0.5% preparation. Similar partial protection was reported for a MC1220 IVR in macaques challenged repeatedly with low-doses of RT-SHIV; however, the animals displayed a significant delay in infection between the IVR and placebo groups (482). Unlike MIV-150, MC1220 is not currently in clinical trials.

Like IVRs, long-acting injectable PrEP formulations are designed to provide antiretroviral drug depots for sustained release to eliminate issues with missed pills. Unlike IVRs, injectable PrEP provides a drug depot that cannot be removed, thus guaranteeing

adherence. Currently there are two drugs being explored for long-acting injectable PrEP, the approved NNRTI RPV and the experimental integrase inhibitor CAB (311, 483). Long-acting CAB (CAB LA) has been shown to protect 75% and 100% of macaques from intravaginal and intrarectal challenges, respectively, if plasma drug concentrations were above a defined protective threshold (484, 485). To elucidate the protective threshold of CAB LA, the drug was administered to macaques that were then repeatedly challenged intrarectally with low doses of SHIV (484). As drug concentrations waned, the authors analyzed plasma concentrations at time of infection and determined that 4-fold the $PAEC_{90}$ of infections provided complete protection with decreasing efficiency as plasma drug concentrations declined below 3-fold the $PAEC_{90}$ (484). The authors also showed that the plasma concentrations needed to reach the protective threshold were obtainable in humans (484), thus providing a realistic drug concentration target for clinical trials. This diligence may provide improved outcomes in clinical trials because dependence on *in vitro* effective values does not necessarily translate to efficacy *in vivo* due to the complex interaction of drug and host factors.

Despite the benefits of testing PrEP agents and modalities in animals prior to clinical trials, not all animal models translate to human success. Because of the favorable safety profile of TFV and success as PrEP in macaques, it was formulated into a well-tolerated 1% vaginal gel to be marketed as a female-controlled prophylactic agent (486). Before movement into efficacy trials, the gel was tested in pigtailed macaques due to similarities between the animal and human menstrual cycles (443). Application of the gel 30 minutes prior to virus challenge protected every animal from 20 separate low-dose vaginal challenges, and the same result was seen with a 1% TFV/5% FTC gel. However, reduced protection in the same model was reported when the gel was applied 3 days before challenge, indicating that the gel has a multi-day effective window

but would be most efficient prior to intercourse (444). Of note, 1% TFV gel was shown to considerably reduce rectal vaginal infections from challenge with SHIV (435, 442, 448, 450). Unfortunately, the CAPRISA 004 clinical trial was unable to repeat the success reported in the macaque models not because the gel was ineffective but rather because of participant adherence (18). A similar discrepancy was seen with carrageenan, a small molecule that blocks viral entry and the active ingredient of Carraguard. Despite success predicted by macaque modeling for a vaginal gel containing Carraguard (470), significant protection was not reported in a clinical trial despite lower infection rates in the Carraguard group (326). This was attributed to low adherence and unreliable monitoring (326). Dissimilarities in success between macaque and human studies emphasize the caution that must be exercised when interpreting results from animal models because of factors such as adherence, which cannot be controlled in human trials.

An example of the utility of macaque models and their necessity in PrEP research is highlighted by the failed microbicide nonoxynol-9, a widely available spermicide. Clinical trials of nonoxynol-9 formulated as a vaginal gel revealed that greater use of the product led to a significant increase in HIV-1 infection attributed to lesions caused by the product (330). Irritation and disruption of macaque vaginal epithelium and microflora by nonoxynol-9 had been previously reported (487) but was not referenced in the clinical trial publication. Later, nonoxynol-9 gel showed a similar disruptive effect from repeated exposure in macaque rectal tissue, which confirmed side effects reported in humans (488). Had the available animal safety data been heeded and the gel tested in a repeated dose-challenge model in macaques, it is likely the product would have been shelved before moving into clinical trials, thus saving resources and avoiding an undesirable outcome.

Aside from testing dosing strategies and efficacy of PrEP, NHPs can be used to model the consequences of drug resistance. Because the current PrEP candidates are also prescribed for ART, there is potential that PrEP rollout could increase prevalence of circulating drug-resistant HIV-1 by selecting mutations post-infection or failing to inhibit drug-resistant transmissions. Within two studies 12 macaques became infected while receiving oral Truvada PrEP, but only two animals developed FTC-associated resistance mutations (M184I/V) despite continued treatment for over 9 weeks post-infection for all animals (316, 436). Resistance was not detected in infected macaques receiving oral TFV for >20 weeks post-infection (433), nor was drug resistance selected during TFV vaginal gel, rectal gel, or IVR PrEP studies with continued application of the products post-infection (442, 444, 449). Despite a small sample size, few instances of resistance selection in macaques were reflected in human trials where resistance development was rare in individuals infected after starting PrEP (320).

A similar issue is the selection of resistance mutations in HIV-1^{+} individuals given PrEP, an event that has been realized in clinical trials albeit in a small number of participants (318-320). This scenario is modeled by simply providing monotherapy to infected animals (389, 465, 489, 490), but the PrEP delivery method needs to be considered. An excellent example is a study that compared resistance selection by systemic versus local PrEP (491). Daily IM injections of MIV-150 provided systemic concentrations of the compound and selected for known NNRTI resistance mutations (*e.g.*, K101E, K103N, Y181C) in 100% of RT-SHIV infected macaques; however, localized drug release from an IVR did not select for mutations in plasma or LNs in the majority of animals (491). The mutations found in the IVR-treated animals were detected at low prevalence and were transient as opposed to the systemic animals that showed steady maintenance of the mutant populations (491). These results demonstrate that the mode of PrEP

delivery can affect resistance selection, and the consequences of infection during PrEP should be properly explored in animal models prior to clinical trials.

The transmission of drug-resistant HIV-1 is another concern with PrEP. It is possible that drug concentrations able to inhibit wild-type (WT) virus may not be effective at blocking drug-resistant virus infection. The only way to ethically explore drug-resistant virus transmission is to treat animals with PrEP and then challenge with drug-resistant isolates. This scenario was first modeled in newborn macaques given SC TFV PrEP (430). The macaques were challenged orally with SIV possessing the K65R TFV-resistant mutation. Infection was prevented in 40% of animals; although, PrEP-treated animals displayed better growth and significantly delayed disease course compared to untreated controls (430). High-level of protection from K65R SHIV infection in a vaginal microbicide model was also reported (450). Two studies using an intermittent oral Truvada strategy showed that PrEP still provides protection against repeated rectal challenges of SHIV with Truvada-resistant mutations; although, the lack of infection is believed to be a result of reduced fitness of the drug-resistant mutants (438, 440, 492). These models provide evidence that PrEP may not increase the prevalence of select transmitted drug-resistant mutations; however, drug-resistant virus challenges should be investigated for each PrEP agent and modality.

NHPs occupy a critical role in the pipeline of PrEP development because they are excellent models for predicting the efficacy and consequences of PrEP. With rigorous animal modeling, ineffective compounds such as nonoxynol-9 can be shelved, or successes like Truvada can be moved into human trials. For example, a variety of experimental entry inhibiting molecules and antibodies has been modeled in macaques to demonstrate *in vivo* effectiveness as microbicides that could be used in future PrEP trials (493-499). Although there is no recognized

standard macaque model for PrEP research, macaques are invaluable for testing dosing strategies, efficacy, and resistance selection and should not be overlooked in future PrEP work.

1.2.4.2 BLT mouse models for PrEP

A less expensive alternative to NHPs for *in vivo* PrEP research is the humanized mouse model. Although a variety of immunodeficient genetic lines of mice and humanization methods are available for HIV-1 research (390, 421), the BLT model is favored for PrEP studies because animals display excellent human immune cell reconstitution in vaginal and rectal tissues (315, 417-419), the main sites of HIV-1 transmission (454). As described above, these mice are generated by transplantation of human fetal liver and thymus tissues under the kidney capsule of immunodeficient mice followed by an injection of autologous human $CD34^+$ stem cells (413-415). BLT mice have been successfully employed in PrEP studies with FDA-approved NRTIs, NNRTIs, and integrase inhibitors (Table 3).

Like macaques, the BLT mouse model has been used to demonstrate the protective efficacy TFV-based PrEP strategies. Daily FTC and TFV displayed protection from vaginal and rectal HIV-1 transmission in BLT mice (315, 500, 506). Although the drugs were not administered orally, the inhibition of HIV-1 infection in mice provided a second pre-clinical *in vivo* model for the utility of systemic FTC and TFV as PrEP. To further validate the BLT model, a study was performed to emulate the PrEP methods tested in a microbicide trial (18). Over 88% of mice treated vaginally with 1% TFV gel in a similar timeframe recommended for women in the CAPRISA 004 trial were protected from HIV-1 infection (500). The same formulation also showed near complete protection from rectal HIV-1 infection when applied 30 minutes prior to challenge (507). The high rate of protection in mice (500, 507) and macaques (443, 444) is likely

Table 3. BLT humanized mouse studies using approved ART as PrEP to prevent mucosal infection by HIV-1

PrEP agent (dose)	Dosing	Challenge virus	Challenge route	Outcome	Ref.
FTC (3.5 mg) & TFV (5.2 mg)	daily IP dose 2 d pre- and 5 d post-challenge	HIV-1_{JR-CSF}	vaginal	5/5 protected	(315)
TFV (1% gel)	dosing 4 h pre- and 4 h post-challenge	HIV-1_{JR-CSF}	vaginal	7/8 protected	(500)
FTC (28 μM) & TFV (16.5 μM) gel	single dose 30 min pre-challenge	HIV-1_{JR-CSF}	vaginal	8/9 protected	(500)
RPV nanoparticle gel (17.5 μg gel)	single dose 1.5 or 25 h pre-challenge	HIV-1_{RHPA}	vaginal	4/4 protected at 1.5 h challenge 4/8 protected from 24 h challenge	(501)
RPV LA (15 mg)	single IM dose 1 week pre-challenge	HIV-1_{CH040}	vaginal	6/6 protected	(501)
RPV LA (15 mg)	single IM dose 1 or 4 weeks pre-challenge	HIV-1_{CH040} HIV-1_{RHPA} HIV-1_{JR-CSF} HIV-1_{THRO}	vaginal	7/10 protected from 1st challenge 3/7 protected from 2nd challenge	(501)
RAL LA (300 mg/kg)	single SC dose 1 or 4 weeks pre-challenge	HIV-1_{CH040} HIV-1_{THRO}	vaginal	6/6 protected from 1st challenge 3/5 protected from 2nd challenge	(502)
TFV nanoparticle gel (0.1%, 0.5%, & 1.0%)	single dose 4 h pre-challenge (0.1%) 1 d pre-challenge (0.5%) 7 d pre-challenge (1.0%)	HIV-1_{WITO} HIV-1_{SUMA}	vaginal	4/4 protected with 0.1% 6/6 protected with 0.5% 0/5 protected with 1.0%	(503)

Table 3 continued

TFV & EVG nanoparticles (200 mg/kg each)	single SC dose 4 or 14 days pre-challenge	HIV-1_{WITO} HIV-1_{SUMA}	vaginal	5/5 protected at day 4 3/5 protected at day 14	(504)
TFV (20, 50, 140, or 300 mg/kg)	daily IP dose 3 d pre- and 4 d post-challenge	HIV-1_{JR-CSF}	vaginal	7/14 protected at 20 mg/kg 8/12 protected at 50 mg/kg 11/13 protected at 140 mg/kg 11/11 protected at 300 mg/kg	(505)
FTC (3.5 mg) & TFV (5.2 mg)	daily IP dose 3 d pre- and 4 d post-challenge	HIV-1_{JR-CSF}	rectal	9/9 protected	(506)
TFV (1% gel)	single dose 30 min pre-challenge	HIV-1_{JR-CSF} HIV-1_{THRO}	rectal	11/12 protected from HIV-1_{JR-CSF} 6/6 protected from HIV-1_{THRO}	(507)
FTC (3.5 mg) & TFV (5.2 mg)	daily IP dose 3 d pre- and 4 d post-challenge	HIV-1_{JR-CSF}	oral	5/5 protected	(508)

FTC, emtricitabine; TFV, tenofovir; RPV, rilpivirine; RAL, raltegravir; EVG, elvitegravir; IM, intramuscular; IP, intraperitoneal; SC, subcutaneous

attributable to the guaranteed adherence of the animals, which is impossible to enforce in humans; however, these distinct animals models suggest that 1% TFV gel should be an effective PrEP strategy if used properly, a concept demonstrated by increased protection rates in participants considered to be adherent in the CAPRISA 004 study (444).

Long-acting injectable formulations of RPV and the integrase inhibitor raltegravir (RAL) have also been tested in BLT mice. IM and SC injections of RPV and RAL, respectively, were given to mice one week prior to intravaginal challenge with transmitted/founder strains of HIV-1 (501, 502). Three separate experiments showed 70-100% protection from HIV-1 transmission with either drug; however, efficacy decreased for both drugs by 4 weeks post-dosing as nearly half the mice became infected after a follow-up challenge despite the presence of drug above defined protective concentrations (501, 502). Lack of protection from the second challenges is likely a result of waning drug concentrations, which reinforces the need for regular dosing to maintain protective concentrations of HIV-1 inhibitors. For both studies, the drug doses administered to mice provided drug concentrations near or above biologically relevant concentrations measured in humans. While concentrations of RAL in mice were similar to those reported in humans (502), the sustained protective plasma concentrations of RPV reported in mice (501) may not be attainable in a clinical setting (338, 340, 348). The disparity between protective concentrations of RPV in mice and in humans highlights the need to use well-characterized, biologically relevant animal models for pre-clinical studies.

The concern over PrEP selection of drug resistance has not been directly explored in humanized mouse; however, resistance mutations were not detected by population-based sequencing in the few breakthrough infections reported to occur in mice treated with FTC and TFV (506), TFV gel (507), RPV LA (501), or RAL LA (502). Inhibition of drug-resistant HIV-1

transmission has not been addressed in BLT mouse models either; although, one study did report inefficient vaginal infection with TFV-resistant virus in BLT mice (509) similar to what was shown in macaques (440, 510)

Overall, results of BLT mouse studies with approved ART drugs show near complete protection when the animals are treated and challenged to reflect recommended use of PrEP in humans (Table 3). These outcomes are similar to the results of macaque studies and demonstrate the utility of the humanized mouse as an *in vivo* model for HIV-1 PrEP research. As in macaques, novel PrEP strategies such as experimental NRTIs (511), peptide and small molecule entry inhibitors (500), CCR5 and viral protein small interfering RNA (siRNA) (512), transgenic immunoglobulin A expression vectors (513), lymphocyte function-associated antigen 1-targeting liposomal CCR5 siRNA nanoparticles (514), and an adeno-associated virus vector for broadly neutralizing antibody production (515) have been tested in BLT mice to show successful inhibition of HIV-1 infection and potential as future PrEP strategies. Despite ongoing research to improve human immune cell function in mice to expand their utility for HIV-1 research (424, 516), humanized mice are a cost-effective alternative to NHPs for PrEP studies.

2.0 SPECIFIC AIMS

Without an effective vaccine to prevent HIV-1 infection, PrEP is currently an effective option to reduce global HIV-1 transmission. Clinical trials have shown that daily oral Truvada is effective at preventing HIV-1 transmission in high-risk populations and has been approved for clinical use. However, PrEP is most efficacious if individuals are adherent to the prescribed regimen. In an effort to improve PrEP adherence, a long-acting injectable formulation of the antiretroviral drug RPV was developed to provide sustained drug concentrations via monthly dosing. Because RPV has a similar resistance profile to other clinically-approved NNRTIs, there is potential that rollout of RPV LA PrEP could increase the prevalence of circulating drug-resistant HIV-1, thus excluding the use of NNRTI-containing ART in newly infected persons. To address resistance concerns with RPV LA PrEP, the first two Aims focus on the selection and transmission of HIV-1 drug resistance in animal models. The third Aim focuses on the *in vitro* resistance profiles of novel RPV analogs that could be further investigated as ART or PrEP.

AIM 1: Study the selection of RPV resistance by RPV LA monotherapy in a RT-SHIV macaque model. The standard of HIV-1 care is to provide ART that contains multiple antiretroviral drugs with distinct resistance profiles to preserve viral suppression in the event of DRM development. Individuals who become infected with HIV-1 prior to or after receiving RPV LA may be more likely to select for DRMs due to RPV monotherapy. Because it is unethical to test resistance selection by RPV LA in HIV-1^{+} individuals, we investigated whether RPV LA

could select for drug resistance in RT-SHIV-infected pigtailed macaques. We hypothesized that RT-SHIV plasma viremia would rebound after RPV LA dosing, and the virus population would contain RPV-associated resistance mutations in the HIV-1 RT coding region.

AIM 2: Investigate the efficacy of RPV LA to prevent mucosal transmission of HIV-1 with NNRTI-resistant mutations in humanized mice. Because of the increase in global ART, prevalence of NNRTI-associated resistance mutations is increasing. It is possible that an individual receiving RPV LA PrEP could be exposed to HIV-1 with a RPV-associated DRM. In this event, the likelihood of RPV LA to prevent transmission of the drug-resistant virus is unknown. To test the efficacy of RPV LA to inhibit vaginal transmission of NNRTI-resistant HIV-1, we challenged humanized mice with two drug-resistant HIV-1 clones that had either low- or high-levels of RPV resistance and compared them to mice challenges with WT HIV-1. We hypothesized that biologically relevant concentrations of RPV LA would inhibit infection by WT HIV-1 but not drug-resistant isolates.

AIM 3: Determine the *in vitro* resistance profile of mutations in HIV-1 selected by novel RPV analogs. HIV-1 has a high mutation rate and can develop resistance in the presence of antiretroviral drugs. However, cross-resistance between NNRTIs can limit ART options in individuals failing therapy. New antiretroviral compounds that are effective against HIV-1 with known DRMs must be discovered. Given the favorable potency and safety profile of RPV, analogs with changes to its structure were developed to inhibit HIV-1 with known NNRTI-associated DRMs. To understand the mechanism of actions of three RPV analogs, HIV-1 was selected in the presence of the compounds to identify novel DRMs. We hypothesized that the analogs would select for novel resistance mutations that would not display cross-resistance to other NNRTIs.

3.0 AIM 1: LOW FREQUENCY OF DRUG-RESISTANT VARIANTS SELECTED BY LONG-ACTING RILPIVIRINE IN MACAQUES INFECTED WITH SIMIAN IMMUNODEFICIENCY VIRUS CONTAINING HIV-1 REVERSE TRANSCRIPTASE

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3.1 PREFACE

Results of this work were partially presented as a poster titled “Selection of drug resistance in a reverse transcriptase simian-human immunodeficiency virus macaque model using long-acting rilpivirine” at the 2014 International Workshop on Antiviral Drug Resistance in Berlin Germany.

This work, essentially as presented here, was originally published in the Journal of Antimicrobial Agents and Chemotherapy (copyright © American Society for Microbiology) as: Melody K, McBeth S, Kline C, Kashuba AD, Mellors JW, Ambrose Z. 2015. Low Frequency of Drug-Resistant Variants Selected by Long-Acting Rilpivirine in Macaques Infected with Simian Immunodeficiency Virus Containing HIV-1 Reverse Transcriptase. *Antimicrob Agents Chemother* 59:7762-7770. Permission to reprint this work was granted by the American Society of Microbiology (http://journals.asm.org).

The work presented in this chapter is for completion of Aim 1 in partial fulfillment of the requirements for the degree of Doctor of Philosophy. Kevin Melody processed blood samples, sequenced HIV-1 clones from plasma vRNA, and performed drug susceptibility assays. Chris Kline isolated vRNA from plasma samples and performed quantitative reverse transcriptase PCR (qRT-PCR) on plasma and tissue samples. Sarah McBeth sequenced vRNA variants isolated from tissue samples. Angela Kashuba completed the PK analysis of RPV in macaques to determine RPV LA dosing.

3.2 ABSTRACT

PrEP using antiretroviral drugs is effective in reducing the risk of HIV-1 infection, but adherence to the PrEP regimen is needed. To improve adherence, a long-acting injectable formulation of the NNRTI RPV has been developed. However, there are concerns that PrEP may select for drug-resistant mutations during preexisting or breakthrough infections, which could promote the spread of drug resistance and limit options for antiretroviral therapy. To address this concern, we administered RPV LA to macaques infected with RT-SHIV. Peak plasma RPV levels were equivalent to those reported in human trials and waned over time after dosing. RPV LA resulted in a 2-log decrease in plasma viremia, and the therapeutic effect was maintained for 15 weeks, until plasma drug concentrations dropped below 25 ng/ml. RT mutations E138G and E138Q were detected in single clones from plasma virus in separate animals but not consistently, and no resistance mutations were detected in vRNA isolated from tissues. WT and E138Q RT-SHIV displayed similar RPV susceptibilities *in vitro*, whereas E138G conferred 2-fold resistance to RPV. Overall, selection of RPV-resistant variants was rare in an RT-SHIV macaque model despite prolonged exposure to slowly decreasing RPV concentrations following two injections of RPV LA.

3.3 INTRODUCTION

Despite increasing use of ART, which suppresses viral replication and reduces the risk of HIV-1 transmission, an estimated two million new HIV-1 infections still occur annually worldwide (1). While research continues to define effective, vaccine-elicited protective immune responses, PrEP

has proven effective in reducing HIV-1 transmission. Thus far, clinical trials have exhibited a 44 to 75% reduction in HIV-1 infections in individuals treated with TFV with or without FTC (17, 19-21). These successes led to FDA approval of daily oral TFV-FTC PrEP for high-risk populations.

One prominent issue with PrEP is that efficacy is dependent on adherence. Data from clinical trials has shown that participants with detectable plasma drug concentrations indicative of adherence have reduced risk of HIV-1 infection, while poor PrEP adherence confers little protection (14, 17-19, 517). Although recent work suggests that sexual event-driven TFV-FTC administration can be effective in men (316, 436, 445), sporadic PrEP adherence may lower PrEP efficacy and promote selection of drug-resistant variants. To reduce pill burden, long-acting injectable nanoparticle formulations of the NNRTI RPV and an experimental integrase inhibitor, CAB, have been developed (338, 518). Injectable PrEP formulations create a depot at the injection site and provide drug release for weeks to months, thus eliminating the need for daily drug administration. Long-acting injectable medications have been used successfully to administer antipsychotics, contraceptives, hormone replacements, and cancer treatments (341, 519-521).

RPV is the most recently approved NNRTI for ART in combination with other antiretrovirals in patients with plasma viremia of less than 100,000 copies of HIV-1 RNA/ml (213, 292, 522). Clinical trials have shown RPV-TFV-FTC to be non-inferior to and more tolerable than EFV-based ART (292). During these trials, patients failing RPV-based ART also tended to select unique NNRTI-associated resistance mutations in RT compared with those on an EFV-based regimen (306). RPV LA is an injectable nanoparticle formulation that has been shown to be safe and tolerable, with detectable drug concentrations maintained in plasma and

tissues weeks after a single injection (340, 347, 483, 523). Multiple clinical trials are under way to test safety and acceptability of RPV LA as PrEP in HIV-1-uninfected men and women (523).

Of concern with RPV LA PrEP is the development of drug resistance if an individual with an undetected HIV-1 infection receives PrEP or someone on PrEP becomes infected, resulting in treatment with RPV monotherapy. Additionally, while patients on a pill-based regimen can cease PrEP and rapidly clear the drug, injectable medications will require weeks to reach undetectable concentrations. These situations may increase the selection of DRMs, promoting spread of drug resistance and limiting future ART options through cross-resistance (16). Resistance analyses of breakthrough infections in TFV-FTC clinical trials revealed that selection of DRMs was rare in patients who became infected after receiving PrEP, but DRMs did develop in the few individuals that were HIV-1^{+} before initiating PrEP (318-320).

Although the development of DRMs during TFV-FTC PrEP clinical trials was rare (318-320), there is currently no published data on the resistance outcome of long-acting PrEP as monotherapy. We report here on a pilot study to explore the selection of drug resistance by RPV LA in RT-SHIV-infected macaques. RT-$SHIV_{mne}$ is a chimeric SIV that contains the HIV-1 reverse transcriptase coding region (370, 389). SIV is not susceptible to NNRTIs due to sequence differences within the RT coding region, but NNRTI sensitivity is established by swapping the SIV and HIV-1 RT coding regions (369, 458). RT-SHIV macaque models have been used to study HIV-1 ART, drug resistance, PrEP, and persistence (369, 389, 464, 466, 467, 470, 472, 473, 476, 477, 480, 491, 524-529). In this study, we treated RT-SHIV-infected macaques with RPV LA and measured plasma viremia, drug concentrations, and drug-resistant isolates over 35 weeks. Our data show that viremia was suppressed by RPV LA monotherapy, which rebounded

to pretherapy levels as plasma drug concentrations waned. However, resistance to RPV in RT-SHIV was difficult to select both *in vitro* and *in vivo*.

3.4 MATERIALS AND METHODS

3.4.1 Cell culture and antiretroviral inhibitors

293T (530) and TZM-bl (531, 532) cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals) and 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 0.292 mg/ml of L-glutamine (P-S-G; Life Technologies). This mix of DMEM, FBS, and P-S-G will henceforth be referred to as complete DMEM (cDMEM). GHOST-R3/X4/R5 cells (533) were maintained in the same medium as described above with the addition of 100 μg/ml of G418 (Life Technologies), 100 μg/ml of hygromycin (Life Technologies), and 0.5 μg/ml of puromycin (EMD Millipore). CEMx174 cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 1% P-S-G. All cell lines were incubated at 37°C with 5% CO_2.

RPV was acquired from the National Institute of Health (NIH) AIDS Reagent Program. RPV LA was donated by Janssen Sciences UC Ireland.

3.4.2 Virus production and titer determination

Plasmids encoding HIV-1_{LAI} with silent restriction sites within RT (534) and RT-$SHIV_{mne}$ (389) were used for virus production by transfection into 293T cells with Lipofectamine 2000 (Life

Technologies) and stored at -80°C. Mutations were introduced into the plasmids by site-directed mutagenesis using either the QuikChange II XL kit (Agilent Technologies) or the Q5 site-directed mutagenesis kit (New England BioLabs). Virus was harvested 48 hours after transfection and titered on GHOST cells as previously described with modifications (533). GHOST cells were seeded in a 24-well plate at $2x10^4$ cells/0.5 ml in cDMEM. The following morning, media was removed and cells were exposed to cDMEM with dilutions of virus stock. Cells and virus were incubated for 2 hours, after which virus and media were replaced with fresh cDMEM and allowed to incubate for 48 hours. After 48 hours, cells were trypsinized using 0.25% trypsin-EDTA (Gibco), fixed in paraformaldehyde at a final concentration of 2%, and analyzed for green fluorescent protein expression by flow cytometry on a LSRII flow cytometer (BD Biosciences). Titers of RT-SHIV stocks were determined using TZM-bl indicator cells and β-galactosidase staining as previously described (370, 535).

3.4.3 Animals

Two juvenile pigtailed macaques were housed at the University of Pittsburgh in accordance with American Association of Accreditation of Laboratory Animal Care (AAALAC) standards. All procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee (IACUC). The animals were negative for serum antibodies to HIV type 2, SIV, type D retrovirus, and simian T-lymphotropic virus type 1 at study initiation. Animals were infected IV with $1x10^5$ infectious units (IU) of RT-$SHIV_{mne}$. At 6 and 8 weeks post-infection, animals were treated with 200 mg/kg of RPV LA by IM injection near the scapula. This dosing regimen was determined by prior PK analysis that showed that a single 50 mg/kg dose of RPV LA did not result in plasma drug concentrations above 16 ng/ml within 24 hours of

administration, consistent with our previous observations of rapid metabolism of NNRTIs in pigtailed macaques (data not shown). Blood was drawn under anesthesia weekly or bimonthly. Animals were euthanized at 35 weeks post-infection, and multiple tissues, including axillary LNs and ileum, were immediately flash frozen and stored in liquid nitrogen.

3.4.4 T cell counts and vRNA isolation

Plasma was separated from EDTA-treated whole blood by centrifugation at 400 x *g* for 10 min and stored at -80°C until processed. vRNA was isolated from plasma as previously described (536). Briefly, plasma was mixed with Tris-buffered saline (Sigma) and pelleted by centrifugation at 21,000 x *g* for 1 hour. Virus was then suspended in 3 M guanidinium chloride, 50 mM Tric-HCl (pH 7.6), 1 mM $CaCl_2$, and 1 mg/ml of proteinase K and incubated at 42°C for 1 hour, followed by addition of 5.7 M guanidine thiocyanate, 50 mM Tris-HCl (pH 7.6), 1 mM EDTA, and 600 μg/ml of glycogen and a 5 min incubation at room temperature. RNA was washed with isopropanol and ethanol and samples were suspended in 10 mM Tris-HCl (pH 8). Tissue vRNA isolation was performed as previously described (537).

Peripheral blood mononuclear cells (PBMCs) were isolated from samples using lymphocyte separation medium (Corning) and treated with ACK Lysing Buffer (Life Technologies) and washed with phosphate buffered saline, pH 7.4 (PBS; Life Technologies) to remove red blood cells. PBMCs were stained with NHP T lymphocyte cocktail (BD Biosciences) with antibodies against cluster of differentiation 3 (CD3), CD4, and CD8. $CD3^+CD4^+$, and $CD3^+CD8^+$ cell populations were measured by flow cytometry. Isotype cocktail C (BD Biosciences) was used for isotype controls.

3.4.5 RPV plasma and tissue concentrations

RPV was extracted from monkey plasma using protein precipitation followed by liquid chromatograph-tandem mass spectrometry (LC-MS/MS) analysis. Calibration standards and quality control (QC) samples were prepared in monkey plasma (EDTA) on the day of analysis. Fifty microliters of each plasma sample was mixed with 50 μl of a 50:50 methanol-water mixture containing amprenavir-d4 as the internal standard. Then, 300 μl of methanol was added to each sample. Following vortex and centrifugation steps, the resulting supernatant was transferred to a 96-well plate for LC-MS/MS analysis. Tissues were homogenized in 1 ml of 70:30 acetonitrile–1 mM ammonium phosphate (pH 7.4) with a Precellys hard tissue grinding kit tube (Cayman Chemical), followed by a similar plasma preparation procedure. Tissue weights ranged from 84.2 to 95.4 mg. Analyte concentrations from tissue homogenates were normalized to tissue weight. A tissue density of 1 g/ml was used to convert concentrations into ng/ml. A Shimadzu high-performance liquid chromatography system was used for separation, and an AB SCIEX API 5000 mass spectrometer (AB SCIEX) equipped with a turbo spray interface was used as the detector. The samples were analyzed with a set of calibration standards and QC samples, with a dynamic range of 0.5 to 2,000 ng/ml. The precision and accuracy of the calibration standards and QC samples were within the acceptable range of 15%.

3.4.6 vRNA quantitation

Copies of vRNA were measured by generation of cDNA from vRNA isolated from plasma or tissues by the SuperScript III first-strand synthesis kit (Life Technologies) with the SIV_{gag}-R primer (5’-CACTAGGTGTCTCTGCACTATCTGTTTTG-3’). qPCR was performed on cDNA

using SsoFast probes SuperMix (Bio-Rad), SIV_{gag}-F (5'-GTCTGCGTCATCTGGTGCATTC-3') and SIV_{gag}-R primers, and the SIV_{gag}-probe (5'-FAM-CTTCCTCAGTGTGTTTCACTTTCTCTTCTGCG-3' 6-carboxytetramethylrhodamine dye). CCR5 primers and probe were previously described (537). Reaction conditions were 1 cycle of 95°C for 2 min followed by 45 cycles of 95°C for 15 s and 60°C for 1 min.

3.4.7 vRNA sequencing

The RT coding region was amplified from cDNA generated from vRNA isolated from plasma or tissues using nested PCR. The first round of PCR was performed with the Platinum Taq DNA polymerase high-fidelity kit (Life Technologies), using primers ZA01 (5'-CTAGATCTGAATTTGCCTGCCC-3') and ZA02 (5'-TGTAACAGGAATAGAGTTAGGTCC-3') with the following reaction conditions: 94°C for 2 min; 40 cycles of 94°C for 15 s, 49°C for 30 s, and 68°C for 2 min; and 1 cycle of 68°C for 5 min. DNA was purified using the ExoSAP-IT PCR cleanup kit (Affymetrix) per the manufacturer's instructions. The second round of PCR amplification was performed with the Platinum PCR Supermix kit (Life Technologies), using primers RT19 (5'-GCAAAAGGATTAAAGGGACAA-3') and RT22 (5'-GGGTAATCCAAATTTGAATACCAATCCT-3') with the following reaction conditions: 94°C for 2 min; 26 cycles of 94°C for 15 s, 63°C for 30 s with -0.5°C increments per cycle, and 72°C for 2 min; 15 cycles of 94°C for 15 s, 50°C for 30 s, and 72°C for 2 min; and 1 cycle of 72°C for 5 min. The PCR product was then cleaned using the Wizard SV gel and PCR cleanup system (Promega) and cloned into TOPO vectors using the pCR 2.1-TOPO TA cloning kit (Life Technologies) per the manufacturer's instructions. Bacterial colonies were screened for full-

length RT sequence by PCR. RT-containing TOPO vectors were isolated from overnight bacterial cultures using the QiaPrep Spin Miniprep kit (Qiagen). Sequences were analyzed using Lasergene (DNASTAR).

3.4.8 RT-SHIV *in vitro* resistance selection

CEMx174 cells (538) were infected with WT RT-SHIV at a multiplicity of infection (MOI) of 0.05 in medium and incubated at 37°C for 2 hours. Cells and virus were then resuspended in medium containing 0.1 nM RPV. Cultures were passaged every 2 to 3 days in new medium. If cytopathic effects (CPE) were apparent, vRNA was isolated from culture supernatant using the RNeasy minikit (Qiagen) and sequenced as described above. If the RT sequence from vRNA isolated from the supernatant was WT, then 50 µl of culture supernatant was used to infect fresh CEMx174 cells and the RPV concentration was doubled to begin a new round of selection.

3.4.9 Drug susceptibility assay

Drug susceptibility assays were performed as previously described, with minor modifications (370). TZM-bl cells were seeded at $5x10^3$ cells in 96-well cell culture-treated white plates (PerkinElmer). The following day, virus and serial drug dilutions were prepared in phenol red-free DMEM (Life Technologies) supplemented with 1% P-S-G and 10% FBS or different amounts of human or macaque serum. Medium containing virus (MOI of 0.05) and drug dilutions in a total volume of 0.2 ml was added to each well in triplicate. Wells with virus and no drug were used as 100% infection controls. Plates were incubated at 37°C for 48 hours. Luciferase activity was measured using Britelite Plus reagent (PerkinElmer) on a Luminoskan

Ascent microplate luminometer (Thermo Scientific). Relative light units (RLU) were converted to percent infection by dividing the RLU of each drug dilution by the RLU of the 100% infection control. Wells containing cells with no virus and no drug were used to normalize for background luciferase output. The EC_{50} was calculated using PRISM 6 (GraphPad). Specifically, EC_{50} was calculated by log transforming drug concentrations and using a four-parameter variable slope nonlinear regression for curve fitting analysis.

3.5 RESULTS

3.5.1 RPV LA treatment of RT-SHIV-infected macaques

To assess the potential for selection of drug-resistant virus *in vivo* during RPV LA treatment, two pigtailed macaques were infected with RT-SHIV at week 0. Both animals showed peak viremia between $1x10^6$ and $1x10^7$ copies of vRNA/ml, which declined to a set point of $5x10^5$ to $9x10^5$ vRNA copies/ml (Figure 7). Based on PK results (data not shown), animals were treated with two 200 mg/kg IM doses of RPV LA at weeks 6 and 8 post-infection. Post-treatment, there was an immediate ~2-log decline in plasma viremia, which was maintained at approximately $1.35x10^4$ vRNA copies/ml until 21 weeks post-infection. Viral loads slowly rebounded thereafter to pretherapy set point values (Figure 7). There was a transient decrease in the percentage of $CD4^+$ T cells in both animals after infection, followed by a steady post-treatment increase for the remainder of the study (data not shown). The only adverse events attributed to RPV LA treatment were redness and swelling at the injection sites.

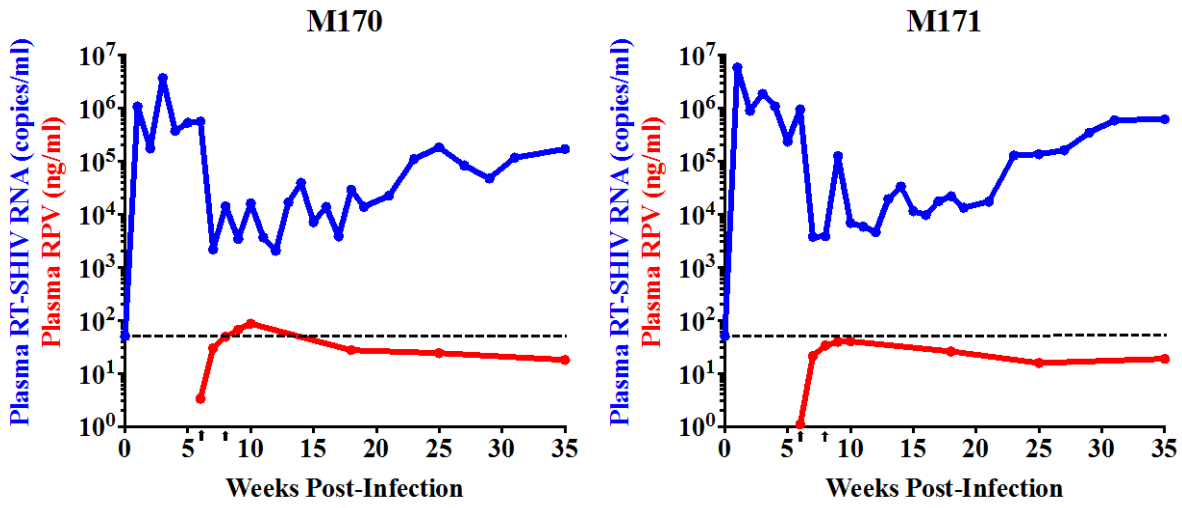

Figure 7. Plasma RT-SHIV levels in two macaques treated with RPV LA monotherapy. Animals were infected at week 0 and received two IM does of RPV LA at 6 and 8 weeks post-infection (designated by arrows under the x axis). Plasma viremia (blue lines) and RPV concentrations (red lines) were measured. The dashed lines indicate the limit of detection of the qRT-PCR assay (50 RNA copies/ml).

Although a standard protective concentration of RPV has not been defined, the *in vitro* EC_{50} of RPV is 0.1 to 0.7 nM (265, 286), and our dosing strategy was designed to achieve plasma RPV concentrations equivalent to that reported for humans: 50 ng/ml (136 nM) (286, 338). Maximum plasma RPV concentrations of 86.5 ng/ml and 40 ng/ml (214.7 nM and 99.3 nM) were detected at week 10 post-infection in animals M170 and M171, respectively (Figure 7). Plasma RPV concentrations were greater than 15 ng/ml (40.9 nM) in both animals up to 29 weeks after the first RPV LA injection. Virus rebound appeared to be associated with declining drug concentrations, although there was not a significant correlation between plasma RPV concentrations and viral loads by linear regression (R^2 = 0.15). RPV concentrations in the axillary LN and ileum were 4- to 421-fold higher than in plasma at the time of necropsy (week 35 post-infection) (Table 4). As tissue homogenate concentrations are averaged across a heterogeneous matrix, these data do not differentiate between extracellular or intracellular drug

exposure and intracellular drug exposure between cell types. Therefore, the averaged concentration could significantly over represent the concentration of drug in mononuclear cells. Since it is difficult to isolate mononuclear cells from tissue without extracellular leaching of drug during the isolation process, intracellular drug concentrations in tissues, and the resulting pharmacodynamic effect, are an area of continued study.

Table 4. RPV concentrations (ng/ml) in plasma and tissues of both animals at 35 weeks post-infection

	RPV concentrations (ng/ml)	
Site	Animal M170	Animal M171
Plasma	18	18
Axillary LN	155	7,590
Ileum	136	80

3.5.2 Plasma RT-SHIV drug resistance detection

To identify selection of RPV-resistant variants, we sequenced the RT coding region of plasma virus at week 6 prior to therapy and at weeks 9, 13, 21, and 25 post-infection. At all time points, the full-length consensus RT sequence matched the starting RT sequence, indicating a lack of detectable mutations that constituted more than 20% of the virus population (data not shown). However, partial and full clonal RT regions were sequenced and revealed many minor viral variants in the plasma (Table 5). Based on Poisson distribution, the probability of detecting variants present at 5% frequency in a virus population with 90% certainty requires analysis of at least 45 clones. Hence, approximately 45 clones were sequenced at 6, 9, 13, and 25 weeks post-infection. The majority of clonal sequences was either WT or had mutations in RT amino acids 1 to 250 that are not associated with known drug resistance. E138G was identified in a single clone at both weeks 9 and 13 in animal M170 but was not detectable later. E138Q was identified in a

single clone at week 25 in animal M171. T69A and K65R are NRTI-associated resistance mutations that were also identified, but they were also detected at frequencies as low as E138G/Q.

To determine if E138G or E138Q conferred RPV resistance in RT-SHIV, the mutations were made in both HIV-1 and RT-SHIV and were tested for susceptibility to RPV compared to that of WT viruses *in vitro*. WT HIV-1 and RT-SHIV displayed similar RPV susceptibilities, with EC_{50}s of 0.67 ± 0.08 and 0.35 ± 0.06 nM (Table 6). E138G conferred a slight increase in EC_{50} compared to WT virus in both HIV-1 and RT-SHIV backbones: EC_{50}s of 0.94 ± 0.03 and 0.76 ± 0.12 nM (1.4- and 2.2-fold changes), respectively. Conversely, E138Q conferred a 4.3-fold EC_{50} increase above WT HIV-1 (2.85 ± 0.08 nM) and no difference from WT RT-SHIV (0.30 ± 0.05 nM). These EC_{50}s for WT, E138G, and E138Q HIV-1 were similar to values previously reported (265, 284).

3.5.3 Tissue RT-SHIV drug resistance detection

Because no consistent RT mutations were identified in plasma virus from either animal, vRNA isolated from axillary LN and ileum obtained at 35 weeks post-infection was clonally sequenced. Axillary LNs were chosen because they were the lymphoid tissues closest to the injection site. Tissues of the gastrointestinal tract were also sampled because they are known to harbor large numbers of infected cells, particularly in the terminal ileum (150). High levels of RT-SHIV RNA copies were measured by qRT-PCR in both the axillary LN and ileum taken at necropsy from both animals (Table 7). While measurements were not taken prior to drug exposure or during peak plasma drug concentrations, the results suggest that significant viral transcription occurs in the LN and ileum despite high RPV levels at those sites (Table 4).

Table 5. Mutations identified in RT of plasma RT-SHIV RNA

	Mutations in RT-SHIV RT[a] (No. of clones)				
Animal	Week 6[b]	Week 9	Week 13	Week 21	Week 25
M170	WT (14) Non-DRM (31)	WT (25) Non-DRM (19) R125G, E138G (1)	WT (19) Non-DRM (25) S68G, E138G (1)	WT (4) Non-DRM (6)	WT (24) Non-DRM (19) K65R (1)
M171	WT (22) Non-DRM (23)	WT (26) Non-DRM (19)	WT (24) Non-DRM (20) T69A (1)	WT (5) Non-DRM (5)	WT (21) Non-DRM (19) E138Q, P157-, L213F, V244C (1)

[a] Results represent a mix of full-length and partial (amino acids 1-250) RT sequences.
[b] Prior to RPV-LA administration (6 and 8 weeks post-infection).
WT, wild-type; non-DRM, mutations not associated with drug resistance; -, codon deletion; underline, known RPV-associated resistance mutations.

Table 6. RPV susceptibility of HIV-1 and RT-SHIV with select mutations identified in plasma and tissue vRNA

Virus	Mutation(s) in RT sequence	EC_{50} (nM)[a]	Fold-change from WT virus[b]
HIV-1	WT	0.67 ± 0.08	1
	E138G	0.94 ± 0.03	1
	E138Q	2.85 ± 0.08	4
	A33V	0.42 ± 0.07	0.6
	Y181H	0.17 ± 0.02	0.3
	A33V, Y181H	0.13 ± 0.01	0.2
RT-SHIV	WT	0.35 ± 0.06	1
	E138G	0.76 ± 0.12	2
	E138Q	0.30 ± 0.05	0.8

[a] Values represent mean and standard deviation of 3 independent experiments, each performed in triplicate.
[b] Fold-change is calculated as the ratio of the mean EC_{50} of mutant to WT virus.

Table 7. RNA copies of RT-SHIV *gag* and macaque *CD4* measured from tissue RNA

		Number of copies (x 10^6)[b]		
Animal	Tissue[a]	*gag* copies	*CD4* copies	*gag*/*CD4* Ratio
M170	Axillary LN	28 ± 0.5	327 ± 10	0.09
	Ileum	334 ± 36	126,237 ± 58,548	0.003
M171	Axillary LN	10 ± 0.8	29 ± 2	0.3
	Ileum	7,863 ± 367	54,343 ± 493	0.1

[a] Tissues taken at 35 weeks post-infection.
[b] Mean ± standard deviation, measured by qRT-PCR in duplicate.

Table 8. Mutations identified in RT of RT-SHIV RNA isolated from tissues

Animal	Tissue[a]	Mutations in RT-SHIV RT[b] (No. of clones)
M170	Axillary LN	WT (1)
		A33V (10)
		M16I, A33V (2)
		A33V, I135T (2)
		A33V, V179A (1)
		A33V, I195V, R206G (1)
		Q85R, H96R (1)
		P217S (1)
		K220E (1)
	Ileum	WT (8)
		A33V (10)
		A33V, D218G (1)
		A33V, V245A (1)
M171	Axillary LN	WT (12)
		N54S (1)
		D76G (1)
		E79G (1)
		G112D (1)
		G155R (1)
		S163G, G196E (1)
		H208R, D250G (1)
		G242. (1)
	Ileum	WT (14)
		A33V, Q145R (2)
		A33V, K173R (1)
		A33V, Y181H (2)
		A33V, T200A (1)

[a] Tissues taken at 35 weeks post-infection.
[b] Sequences represent amino acids 1-250 of RT; WT, wild-type; ., stop codon

No known DRMs were detected in 20 clones from either tissue from both animals (Table 8). However, the A33V mutation was identified in 45% of ileum clones from both animals and 80% of axillary LN clones from animal M170. This mutation also was identified in a single clone in the plasma for M170 at week 9 (Table 5). Y181H was present and linked to A33V in two clones from the ileum of animal M171. While Y181H has not previously been reported in association with NNRTI resistance, Y181C, Y181I, and Y181V mutations display RPV resistance (265). Interestingly, we detected G112D in a single clone obtained from one axillary LN from animal M171. This mutation confers 2-fold resistance to RPV and was selected by an RPV analog *in vitro* (see Chapter 5).

Due to the high frequency of the A33V mutation in the viruses isolated from tissues and the association of RPV resistance with mutations at position 181, the amino acid substitutions A33V and Y181H were made separately and together in HIV-1 and tested for RPV sensitivity. A33V did not confer RPV resistance, while Y181H alone or with A33V conferred hypersusceptibility to RPV compared to WT HIV-1: 0.3- and 0.2-fold, respectively (Table 6).

3.5.4 Effects of human and macaque plasma on RPV inhibition of HIV-1 and RT-SHIV

As RPV inhibition of HIV-1 was greatly decreased by the presence of high human serum proteins *in vitro* (265), the effect of macaque serum on RT-SHIV inhibition by RPV was investigated in cell culture. Similar to previously published results in which 50% human serum increased the EC_{50} of RPV against HIV-1 18.5-fold (265), 25% human serum reduced RPV inhibition of HIV-1 *in vitro* in our study 12-fold (Table 9). Similarly, addition of macaque serum increased the EC_{50} of RPV against RT-SHIV compared to the values with medium with FBS

(Table 9). The results suggest that macaque serum proteins do not impact RPV activity against RT-SHIV more than human serum proteins.

Selection of RPV resistance in RT-SHIV was also difficult to achieve in CEMx174 cells using the same virus stock as used to infect the macaques. RT-SHIV-infected cells were exposed to increasing concentrations of RPV for 350 days (0.1 to 409.6 nM), and vRNA isolated from supernatants from all time points had WT sequence in the RT coding region (data not shown). This is in contrast to selection of Y181C or K103N in RT-SHIV in CEMx174 cells by NVP or EFV, respectively (370).

Table 9. Effect of human and macaque serum on RPV EC_{50}

Virus	Serum	EC_{50} (nM)[a]	Fold-Change[b]
HIV-1	10% FBS	0.54 ± 0.05	1
	5% Human	1.34 ± 0.10	3
	10% Human	2.45 ± 0.44	5
	25% Human	6.72 ± 0.39	12
RT-SHIV	10% FBS	0.42 ± 0.08	1
	5% Macaque	0.45 ± 0.21	1
	10% Macaque	1.16 ± 0.15	3

[a] Mean ± standard deviation of triplicates from one experiment.
[b] Compared to assay performed with 10% FBS.

3.6 DISCUSSION

Although global HIV-1 incidence is decreasing in large part due to rollout of ART, an effective vaccine or cure is not yet available. In lieu of a vaccine, FDA-approved PrEP comprised of two NRTIs can reduce infections in high-risk populations (17-21). However, the most significant barrier to PrEP efficacy is patient adherence, as >90% protection is observed in patients with consistently detectable plasma drug concentrations, whereas no protection is seen in participants

showing undetectable drug concentrations (14, 15, 17, 21, 517, 539). To improve adherence, a long-acting injectable nanoparticle formulation of RPV has been developed and is currently being evaluated in clinical trials (523). However, inappropriate use of PrEP by suboptimal dosing or in individuals who are HIV-1^{+} may lead to development of drug resistance. The development of DRMs during PrEP could limit future therapy options, as was the case in single-dose NVP trials to prevent mother-to-child transmission of HIV-1 (540).

As there are no currently published data on the effect of RPV LA on HIV-1 resistance selection, we designed a pilot study to explore whether RPV LA monotherapy could select for drug resistance in a preexisting RT-SHIV infection in macaques. Although untreated macaques were not included as a comparison control, the peaks and set points of plasma viremia in the two animals in this study were similar to those in our previous study (389). While RPV LA dosing and metabolism in macaques were different than in humans, its administration in this study led to plasma RPV concentrations detected in the animals that were comparable to concentrations reported for humans who received 600 mg RPV LA doses (338, 340). RPV LA treatment displayed a therapeutic effect with approximate 2-log decreases in plasma viremia one week after treatment that was sustained for roughly 15 weeks after the first injection, and viremia increased as plasma RPV concentrations dropped below 25 ng/ml (68 nM). Axillary LN and ileum RPV concentrations were at least 4.5-fold greater than plasma concentrations 29 weeks after the first injection, which is consistent with a previous report showing that RPV LA achieves higher concentrations in LNs than in plasma (347). While tissues were not assessed at earlier time points for vRNA and drug concentrations, it is likely that tissue RPV concentrations were higher at earlier time points, and it is clear that drug concentrations were not sufficient to fully suppress virus replication in tissues or blood, as evidenced by high RT-SHIV RNA levels.

Despite waning RPV plasma concentrations and lack of complete virus suppression, which may be seen in noncompliant individuals and suggesting suboptimal *in vivo* drug inhibition, persistent DRMs were not selected in the plasma or tissues of either animal after RPV LA administration. This is in contrast to our previous studies, in which EFV monotherapy was administered over four days in RT-SHIV-infected macaques and rapidly selected the NNRTI resistance mutation K103N in the plasma virus that affected the efficacy of subsequent combination therapy containing EFV, particularly in two animals with high plasma viremia levels similar to the animals in this study (389, 527, 541). Another study using a different strain of RT-SHIV in rhesus macaques also showed that K103N and other DRMs arise during EFV monotherapy (466). The RT mutation K103N confers approximately 20- to 35-fold resistance to HIV-1 *in vitro* and arises in HIV-1-infected individuals on EFV-based ART (265, 542).

In contrast, K103N and other single DRMs selected by NNRTIs approved earlier than RPV, such as Y181C, confer no or low-level (*i.e.*, 1- to 3-fold) resistance to RPV (265). K103N was not detected in patients failing RPV-containing ART in two clinical trials (306). In fact, with the exception of K101P, Y181I, and Y181V, which confer significant resistance to RPV *in vitro* (52-, 15-, and 12-fold changes above WT HIV-1, respectively), HIV-1 with any other single NNRTI-associated resistance mutation has no or low-level resistance to RPV (265). Unlike K103N and Y181C, the mutations K101P and Y181I/V require at least two base changes to be made and therefore are likely more difficult to develop. In addition, the accumulation of multiple NNRTI resistance mutations, as occurs in treatment-experienced HIV-1^{+} individuals, can enhance RPV resistance (265, 283, 306, 543), which is less likely to occur prior to therapy or to be transmitted to newly infected individuals (285). Thus, it appears that isolates that are highly

resistant to RPV are difficult to develop compared to mutants that arise during use of other NNRTIs, such as EFV and NVP.

Similarly, in a macaque study investigating sustained release of the novel NNRTI MIV-150 from IVRs in RT-SHIV-infected rhesus macaques, DRMs were not detected by clonal sequencing of plasma virus after 42 days in 5/6 animals using the IVR; however, DRM selection did occur when animals were treated systemically with MIV-150 (491). One IVR-treated animal had a single plasma clone containing Y181I, while all other clones were WT. Similar results were found in LNs at day 57, and no DRMs were detected in vRNA isolated from the cervix or vagina, the site of drug release.

Recently, one individual unexpectedly became infected with HIV-1 in the lowest RPV LA dose arm (300 mg) of the SSAT 040 trial (1/66 in the overall study and 1/20 in the 300-mg dose arm), which evaluated the PK of the drug in HIV-1$^-$ individuals (351). K101E was selected in the RT of plasma HIV-1 after seroconversion (*i.e.*, K101E HIV-1 was not transmitted to this individual), and K101E HIV-1 clones had 4-fold resistance to RPV *in vitro* compared to that of WT virus. K101E or E138K/Q was the most common RT mutation detected in patients failing RPV-containing therapy in the ECHO, THRIVE, and STaR studies, with E138K constituting the majority of detected NNRTI-associated resistance mutations (306, 544). Molecular modeling studies of RPV bound to the crystal structure of HIV-1 RT show the formation of a salt bridge between E138 and K101 in the WT p66 subunit (545). This interaction was lost with the substitution of the low-level RPV-resistant mutations E138A/G/K/R/Q or K101E, suggesting that disruption of the K101-E138 salt bridge causes low-level RPV resistance (301). A higher prevalence of E138 mutations, particularly E138K/G, in HIV-1 from individuals failing RPV-containing therapy is unknown but may be due to mutational bias (546) or G-to-A hypermutation

(547). The E138Q/G mutations that were selected by RPV LA in the macaques in our study and the K101E mutation that was selected in the individual who failed RPV LA were likely stochastic events.

While this is a small pilot study, the data are encouraging that drug resistance may be difficult to develop in HIV-1 RT during RPV LA monotherapy compared to NNRTIs approved prior to RPV. The single DRMs that we did detect remained a minor species despite persistent drug concentrations. Future studies looking at a larger group of animals with different viremia levels and comparing daily oral RPV dosing to dosing with RPV LA with different lengths of sustained released are warranted to understand how they influence the development of drug resistance. In addition, the effect of development of minority, low-level RPV-resistant viruses on subsequent combination therapy should be addressed.

4.0 AIM 2: HIGH CONCENTRATIONS OF LONG-ACTING RILPIVIRINE ARE EFFECTIVE AT INHIBITING TRANSMISSION OF LOW-LEVEL DRUG-RESISTANT HIV-1 IN HUMANIZED MICE

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4.1 PREFACE

Results from this research were presented in part as an oral presentation titled “Vaginal transmission of WT or resistant HIV-1 was not inhibited by long-acting rilpivirine at high plasma and genital concentrations in BLT mice” at the 2016 HIV Research for Prevention conference in Chicago, Illinois.

The work presented in this chapter is for completion of Aim 2 in partial fulfillment of the requirements for the degree of Doctor of Philosophy. Kevin Melody performed the *in vitro* virus assays, all *in vivo* work, and population sequencing of breakthrough infection vRNA. Mackenzie Cottrell and Angela Kashuba completed PK analysis of RPV in plasma and tissue samples collected by Zandrea Ambrose and Kevin Melody. Moses Bility and Kevin Melody conducted the surgeries to generate a cohort of BLT humanized mice. Chris Kline performed the plasma vRNA extractions and qPCR assays. Single-genome sequencing was performed by Brandon Keele. Statistical analysis was performed with assistance from Kathleen Shutt.

4.2 ABSTRACT

RPV LA is an injectable long-acting formula of the antiretroviral RPV, which was developed to improve PrEP adherence. To determine whether RPV LA could prevent transmission of RPV-resistant HIV-1, BLT humanized mice were challenged vaginally with WT, low-resistant (Y181C), or high-resistant (Y181V) HIV-1 at days 1 or 7 after RPV LA administration. At day 7 post-RPV LA dosing, drug concentrations in mouse plasma and mucosal tissues were similar to human women given a 1,200 mg dose of RPV LA; whereas, RPV concentrations were >7-fold higher at day 1 post-dosing. Sixty-six percent of mice challenged with WT HIV-1 became infected in both dosing groups versus 86% of untreated mice; however, 0 or 50% of mice became infected with Y181C HIV-1 at days 1 or 7 post-RPV LA dosing, respectively, compared to 63% of untreated mice. In contrast, 88% of untreated and 100% of day 1 post-RPV LA dosing Y181V-challenged mice became infected. A group of mice were challenged with WT HIV-1 at day 7 post-RPV LA dosing to compare efficacy of HIV-1 inhibition via rectal or vaginal challenge. Forty percent of rectally challenged compared to 66% of vaginally challenged mice that received PrEP were infected with WT HIV-1. Population sequencing of plasma vRNA isolated from a subset of mice infected with WT or Y181C HIV-1 did not detect DRMs in RT. We conclude that RPV LA is effective at preventing vaginal transmission of low-level RPV-resistant HIV-1 at plasma and mucosal RPV concentrations that are not achieved in humans. In addition, breakthrough infection in the presence of RPV LA does not readily select for resistance mutations.

4.3 INTRODUCTION

An estimated 2.1 million new HIV-1 infections occurred in 2015 (1). In the absence of a vaccine, daily oral PrEP with Truvada, a combination of FDA-approved NRTIs, is shown to be effective at preventing HIV-1 transmission in animal models (315, 316) and human clinical trials (17, 19, 20, 23). Truvada is currently available as PrEP for high-risk individuals in nearly a dozen nations. Microbicide gels and IVRs containing RT inhibitors have also been shown to be effective at inhibiting HIV-1 transmission (18, 336), but are not yet approved by the FDA. Despite significant rates of HIV-1 inhibition in most PrEP trials, lack of adherence is the biggest barrier to PrEP efficacy (14, 15, 18).

To increase adherence to PrEP, the FDA-approved antiretroviral drug RPV was developed into an injectable long-acting nanoparticle formulation, RPV LA (311, 347). RPV is the most potent approved NNRTI and has a favorable safety and tolerability profile that makes it ideal for nanoparticle formulation (311, 312). The long-acting formulation creates a depot of drug at the site of injection and provides sustained systemic release and maintenance of proposed protective RPV concentrations. RPV LA dosing would eliminate the need for daily Truvada pills, reduce lapse in systemic drug concentrations from missed doses, and allow adherence monitoring by healthcare providers. Phase I clinical trials with RPV LA showed that it is safe and tolerable with only mild reported adverse events (338, 340, 348). PK data reveals that a 1,200 mg dose of RPV LA administered monthly should achieve plasma and tissue RPV concentrations estimated to be effective at inhibiting HIV-1 infection for at least one month post-dosing (338, 340, 348). A Phase II clinical trial to determine long-term safety and tolerability of RPV LA is currently underway (https://clinicaltrials.gov; NCT02165202).

A concern of using drugs approved for both ART and PrEP (*i.e.* Truvada or potentially RPV) is the potential inability of the PrEP agent to prevent transmission of drug-resistant HIV-1. Subsequently, transmitted drug-resistant virus could reduce ART options in newly infected individuals. For example, RPV is a diarylpyrimidine similar to ETR (286). Because of their similar structures, they select for similar DRMs that confer resistance to both drugs (265, 548). Additionally, all currently approved NNRTIs target the same binding pocket in RT (49, 218, 288, 549); hence, some mutations can confer cross-resistance to all NNRTIs (16, 265). With increases in global availability of ART, there is also a significant increase in drug-resistant HIV-1 transmission, particularly NNRTI resistance (285). It is estimated that prevalence of RPV resistance is as high as 3.6% depending on geographic location, but the frequency of DRMs at amino acid 138 of RT can be as high as 6.1% depending on HIV-1 subtype (310). Increasing DRM prevalence could lead to individuals on PrEP being at higher risk of exposure to drug-resistant HIV-1.

To determine if RPV LA can inhibit transmission of drug-resistant HIV-1, we treated BLT mice with RPV LA and then mucosally challenged them with WT or drug-resistant HIV-1. To determine if there is a threshold for which RPV is still protective against RPV-resistant HIV-1, mice were challenged with HIV-1 containing a low-resistant (Y181C) or high-resistant (Y181V) DRM. We chose Y181C because it is the second most prevalent NNRTI-associated DRM (285), while Y181V is one of the few single DRMs that confer greater than 10-fold resistance to RPV (265). Both mutations are also considered surveillance transmitted DRMs (309). Rectal challenges with WT HIV-1 were also performed to compare the ability of RPV LA to inhibit transmission via different mucosal routes. In addition, the RT region of plasma HIV-1 from mice that became infected was sequenced to determine if DRMs were selected by systemic

RPV after breakthrough infection. Our data indicate that RPV LA inhibits vaginal infection with low-level resistant virus but only at high plasma and tissue RPV concentrations not achieved in humans. Our data also suggest that selection of DRMs is unlikely in the event of breakthrough infection during RPV LA PrEP.

4.4 MATERIALS AND METHODS

4.4.1 Cell lines and RPV

293T, TZM-bl, and GHOST-R3/X4/R5 cells were cultured as described in section 3.4.1. Human PBMCs were isolated from whole blood by density gradient centrifugation using lymphocyte separation media followed by ACK Lysing Buffer incubations and PBS washes to remove red blood cells. PBMCs were stimulated for 48 hours in RPMI 1640 with 10% FBS, 1% P-S-G, 50 U/ml interleukin-2 (IL-2; Roche), and 5 μg/ml phytohaemagglutinin (Thermo Fisher). After stimulation, PBMCs were washed and cultured in RPMI 1640 with 10% FBS, 1% P-S-G, and 20 U/ml IL-2. All cell lines and cell culture experiments were maintained at 37º C with an atmosphere of 5% CO_2.

RPV was acquired from the NIH AIDS Reagent Program. RPV LA was obtained from Janssen Sciences UC Ireland.

4.4.2 HIV-1 production

HIV-$1_{NL4-BAL}$ is a chimeric CXCR4-tropic virus with a CCR5-tropic envelope gene (550) and will be referred to as HIV-1. The Y181C and Y181V mutations were generated in a full length HIV-1 proviral plasmid using a Q5 site-directed mutagenesis kit. HIV-1 production and virus titer on GHOST-R3/X4/R5 cells were performed as described in section 3.4.2.

4.4.3 RPV susceptibility of WT and drug-resistant HIV-1

Susceptibility of HIV-1 to RPV or RPV LA was tested as described in section 3.4.9 with minor changes. $1x10^4$ TZM-bl cells/well were seeded in 96-well cell culture-treated white plates. The day following seeding, the cells were incubated with virus at a MOI of 0.02 in media with 10% FBS.

4.4.4 HIV-1 replication assay

PBMCs were infected with WT, Y181C, or Y181V HIV-1 at a MOI of 0.01 and incubated for 2 hours at 37ºC. After the incubation, cells were washed with PBS, resuspended in medium, and incubated at 37ºC. Half the media was replaced every 2 days. During media replacement, the removed supernatants were filtered using a 0.45 μm MCE membrane syringe filter (Argos Technologies) and stored at -80ºC. HIV-1 capsid protein (p24) in the supernatant samples was measured using the HIV-1 p24 ELISA kit (Xpress Bio).

4.4.5 Animals

Female BLT humanized or NSG mice were obtained from Jackson Laboratory and were housed at the University of Pittsburgh Division of Laboratory Animal Resources. All procedures were performed in accordance with AAALAC and were approved by the University of Pittsburgh IACUC. Humanization of NSG mice was performed as previously described (413). Briefly, NSG mice were irradiated with 200 cGy of ^{137}Cs, and fur on the left lateral side was removed the night before surgery. On the day of surgery, mice were anesthetized with 2% isoflurane and injected SC with 5 mg/kg carprofen (Thermo Fischer) and IP with 5 mg/kg ceftiofur (Thermo Fischer). Approximate 1 mm^3 pieces of thawed human fetal liver and thymus tissue were implanted under the left kidney capsule of each mouse. Following suturing, the mice were injected IV with approximately 1.5x10^5 autologous human CD34$^+$ cells. At 12 weeks post-surgery, the mice were assessed for human immune reconstitution. To measure the frequency of human immune cells, blood was collected by submandibular bleeding (551) and PBMCs were isolated and stained for live/dead-APC-Cy7 populations (BD Biosciences) anti-human cluster of differentiation 45 (CD45)-PE (Miltenyi), CD3-V450, CD4-PerCP-Cy 5.5, and CD8-FITC (BD Biosciences). Cell populations were analyzed by flow cytometry using the following gating strategies: lymphocytes (side scatter area by forward scatter area) -> single cells (forward scatter height by forward scatter area) -> live/dead (forward scatter area by APC-Cy7) -> human CD45$^+$ (forward scatter height by PE) -> human CD45$^+$CD3$^+$ (PE by V450) -> human CD45$^+$CD3$^+$CD4$^+$ and CD45$^+$CD3$^+$CD8$^+$ (FITC by PerCP-Cy 5.5). Non-transplanted NSG mice were used as controls and demonstrated no staining for human cell markers. The average human CD45$^+$ cell reconstitution of mice used in this study was 46.2 ± 12.3%.

To determine the PK of RPV LA in female BLT mice, the drug was administered IM into the left gastrocnemius muscle at 150 mg/kg to 14 mice. Blood samples were collected at 1, 2, 6, 12, 24, 48 hours, and 7 days post-injection. At each time-point, two animals were sacrificed and blood, female genital tract, and rectal tissue were harvested.

To determine RPV LA efficacy to prevent HIV-1 transmission, mice were treated with RPV LA as described above. At days 1 or 7 post-RPV LA dosing, mice were challenged vaginally with $1x10^5$ IU of WT, Y181C, or Y181V HIV-1 in <15 μl of PBS. Another cohort was challenged rectally with $1x10^5$ IU of WT HIV-1 at day 7 post-RPV LA dosing. Untreated mice were challenged in the same manner and used as controls. Blood was collected in EDTA-treated tubes (Ram Scientific) by submandibular bleeding, and plasma was separated by centrifugation at 400 x *g* for 10 min and stored at -80ºC until RNA extraction. Mice were considered infected if they had detectable plasma viremia at two time points. PBMCs were isolated from whole blood by two ACK Lysing Buffer incubations and one PBS wash and stored at -80ºC. Blood samples were collected weekly for the first 3 weeks post-challenge and then bi-weekly until 10 weeks post-challenge. Due to the frequency of blood collection, mice were injected IP with pre-warmed PBS after each blood sample for fluid replacement, and DietGel 76A (ClearH_2O) was provided to each cage to improve recovery. After 10 weeks, mice were euthanized and brain, lung, liver, spleen, left kidney with thymus organoid, mesenteric LNs, small intestine, female genital tract, and colon tissue were harvested. If possible, tissue samples were collected in triplicate and flash frozen whole, flash frozen in RNAlater (Ambion), or suspended in 10% buffered formalin (Sigma).

4.4.6 PK analysis of RPV LA

RPV concentrations in plasma and tissue were measured as described in section 3.4.5.

4.4.7 Quantitation of plasma HIV-1 RNA

Plasma HIV-1 RNA was isolated as previously described (552) and quantified by the single copy assay (553). Briefly, avian sarcoma-leukosis virus, kindly provided by Stephen Hughes, was spiked into each plasma sample as an internal RNA isolation control and total viral RNA was isolated with guanidinium isothiocyanate and glycogen. cDNA synthesis was performed with random hexamers. Quantitative PCR was performed in duplicate for all samples and standards.

4.4.8 Sequencing of vRNA

Population sequencing of vRNA recovered from plasma of the mice was performed using nested PCR. cDNA was generated from vRNA using the SuperScript III first-strand synthesis kit with random hexamer primers. The cDNA was first amplified using the primers BAL3F (5'-TGTGGAAAGGAAGGACACC-3') and BAL5R (5'-TCACTATTATCTTGTATTACTACTGC-3') with the following reaction conditions: 94°C for 2 min; 40 cycles of 94°C for 15 s, 49°C for 30 s, and 68°C for 3 min; and 1 cycle of 68°C for 5 min. The second round of PCR amplification was performed using primers LAI-RT-F (5'-TTTGCCAGGAAGATGGAAAC-3') and LAI-RT-R (5'-TCACTAGCCATTGCTCTCCA-3') with the following reaction conditions: 94°C for 2 min; 26 cycles of 94°C for 15 s, 63°C for 30 s with -0.5°C increments per cycle, and 72°C for 2 min; 15 cycles of 94°C for 15 s, 52°C for 30 s,

and 72°C for 2 min; and 1 cycle of 72°C for 5 min. The Platinum Taq DNA polymerase high-fidelity kit was used for both rounds of PCR. PCR products were purified with the Wizard SV gel and PCR cleanup system. The full RT coding region was sequenced using primers LAI-RT-F, 200F (5'-GTAGGATCTGACTTAGAAA-3'), 350F (5'-CAGGAAAATATGCAAGAATG-3'), and LAI-RT-R. Sequences were assembled and analyzed using Sequencher (Gene Codes Corporation) and Lasergene (DNASTAR).

Single-genome sequencing was performed as previously described (554).

4.4.9 Statistical analyses

Fischer's exact test was used to test for efficacy to prevent HIV-1 infection between untreated and RPV LA-treated groups challenged with the same virus. Two-sided log-rank test was conducted to determine differences in time to vRNA detection in plasma samples. Mann-Whitney test was used to analyze differences in plasma viremia. A p value of ≤ 0.05 was considered statistically significant for all tests.

4.5 RESULTS

4.5.1 *In vitro* RPV susceptibility and replication of WT and mutant HIV-1

Mutations at amino acid position 181 in RT have been shown to confer resistance to RPV (265). Site-directed mutagenesis was used to introduce the Y181C (TAT -> TGT) or Y181V (TAT -> GTT) mutations into the HIV-1 proviral plasmid. To confirm resistance of Y181C/V HIV-1,

susceptibility of the WT and mutant viruses to RPV was determined in a single-cycle replication assay. The EC_{50} values of RPV to WT, Y181C, and Y181V HIV-1 were 0.6, 1.2, and 16.4 nM, respectively (Figure 8A). This translated to 2- and 27-fold resistance conferred by Y181C and Y181V mutations, respectively. The fold-change in EC_{50} for Y181C HIV-1 was consistent with previous reports (265, 543), but the EC_{50} for Y181V HIV-1 was twice as high (265). Nonetheless, Y181C and Y181V displayed the desired low- and high-level resistance to RPV.

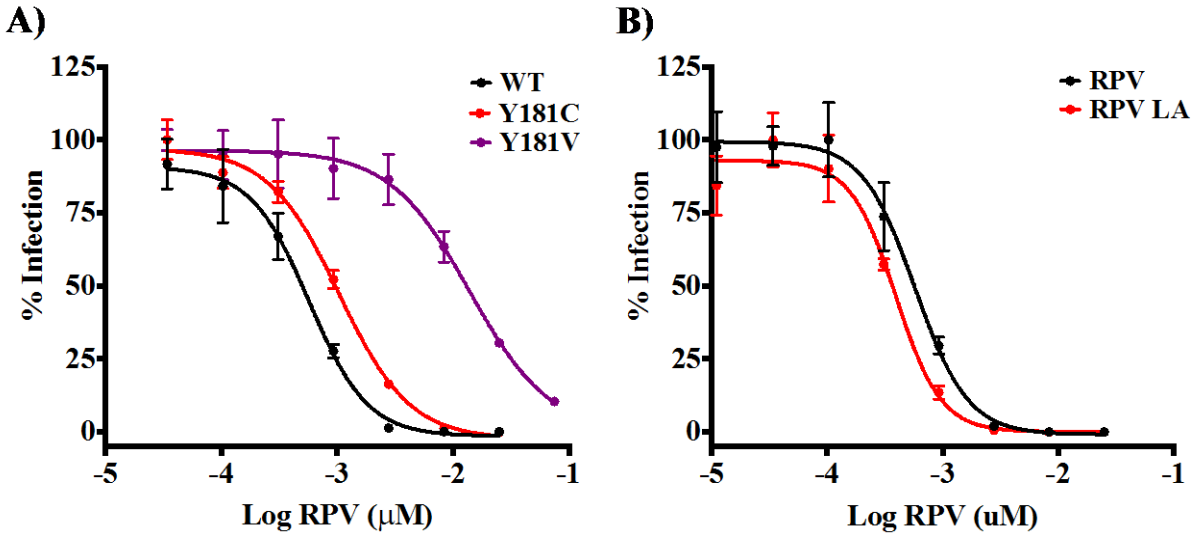

Figure 8. *In vitro* susceptibility of HIV-1 to RPV.
TZM-bl cells were cultured in the presence of HIV-1 and serial dilutions of powder RPV or RPV LA. Luciferase production was used as a measure of the efficacy of drugs to prevent infection. Results are normalized to luciferase production in cells infected with HIV-1, but not treated with RPV. Susceptibility of WT, Y181C, and Y181V HIV-1 to powder RPV was determined and the results are representative of 3 independent experiments each performed in triplicate (A). The potency of RPV LA to inhibit WT HIV-1 infection was compared to powder RPV (B). Error bars represent standard deviation of experimental triplicates.

To confirm potency of our RPV LA stock, WT HIV-1 susceptibility to reconstituted powder RPV (used above) and RPV LA was directly compared (Figure 8B). Both powder RPV and RPV LA showed similar levels of potency to inhibit WT HIV-1 at 0.6 and 0.4 nM, respectively. Thus, our stock of RPV LA should be potent enough to inhibit HIV-1 transmission.

Replication of WT, Y181C, and Y181V HIV-1 was determined in activated human PBMCs *in vitro.* Both mutants had 5-fold or less p24 production at all time points and similar replication kinetics compare to WT HIV-1 (Figure 9). Our results for Y181C virus replication capacity are similar to a slight replication deficiency relative to WT HIV-1 reported in a transformed T lymphocyte cell line (555). Reports directly comparing WT and Y181V HIV-1 replication have not been published. Our *in vitro* data show that our challenge viruses are susceptible to RPV, infectious, and replication competent in primary human cells.

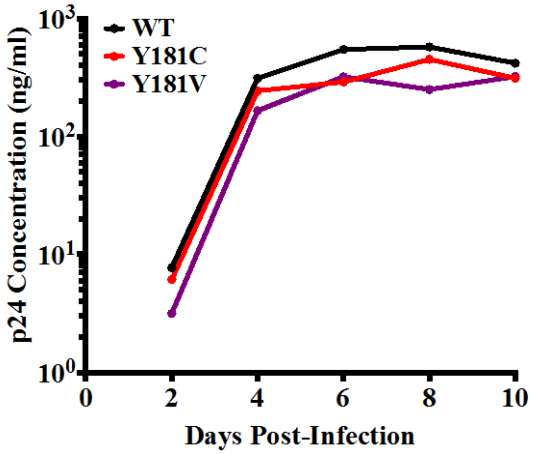

Figure 9. *In vitro* replication kinetics of WT, Y181C, and Y181V HIV-1 in primary human cells.
Human PBMCs were infected with WT, Y181C, or Y181I HIV-1 at a MOI of 0.02. Culture supernatant was measured for virus p24 as a marker for virus production.

4.5.2 Plasma and mucosal tissue PK of RPV LA after a single dose

To assess the PK of RPV, drug concentrations were measured in plasma, female genital tract, and rectal tissues of female BLT mice administered a 150 mg/kg dose of RPV LA (Figure 10). The only side effect noticed was limping directly after the injection. Plasma RPV peaked in the plasma at a mean concentration of 2,277 ng/ml three hours post-injection and declined to a mean

concentration of 130 ng/ml by seven days. The RPV concentrations in female BLT mice at day 7 post-dosing are consistent with peak concentrations reported in human women given a single 1,200 mg dose of RPV LA (338, 340). Mean drug concentrations in female genital tract tissue peaked at 653 ng/ml at 12 hours and declined to a mean of 93 ng/ml at day 7, which is 50% higher than RPV concentrations in women 28 days after a single 1,200 mg dose of RPV LA (338). Concentrations of RPV in rectal tissue varied, likely from the presence of fecal matter in the samples. The mean RPV concentration in rectal tissue at day 7 post-dosing was 37 ng/ml, which is half of the concentration reported in men 14 days after being given a 600 mg dose of RPV LA (338). Mean RPV concentrations measured in the mice were 3- to 11-fold higher than the $PAEC_{90}$ of 12.1 ng/ml reported by Jackson *et al*. (338). Thus, biologically relevant plasma and mucosal tissue concentrations of RPV were achieved in BLT mice at day 7 post-dosing.

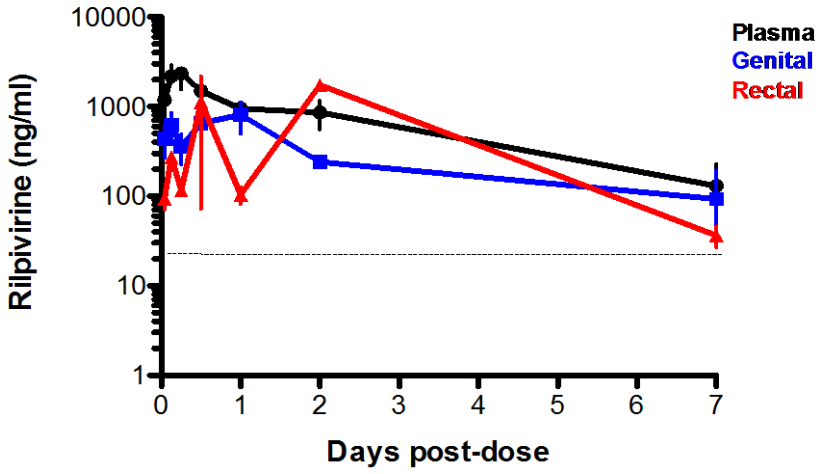

Figure 10. PK profile of RPV LA in BLT mice.
Female BLT mice were administered a single 150 mg/kg IM dose of RPV LA. RPV concentrations were measured in plasma, female genital tract, and rectal tissues at 1, 3, 6, 12, 24, and 48 hours and 7 days post-dosing. The dashed line represents the $PAEC_{90}$ of 12.1 ng/ml, which is the theoretical protection limit of RPV.

4.5.3 Inhibition of HIV-1 vaginal transmission after RPV LA PrEP

To determine the efficacy of RPV LA to inhibit vaginal transmission of HIV-1, control and treated BLT mice were challenged with WT virus one week after receiving RPV LA. Six of 7 (85.7%) and 4/6 (66.7%) mice became infected in the untreated and RPV LA-treated groups, respectively (Table 10). Due to the lack of protection at day 7 post-RPV LA dosing, we hypothesized that the drug concentrations may have been too low to be effective. Thus, we chose to challenge animals when RPV was at higher concentrations. An additional group of animals was challenged with WT HIV-1 at day 1 post-RPV LA dosing when plasma and vaginal drug concentrations were 7- and 9-fold higher, respectively. Again, 4/6 (66.7%) treated mice became infected with WT HIV-1 (Table 10). There was no significant difference in number of infected animals between treated and untreated groups despite RPV concentrations ($p = 0.559$).

We also hypothesized that the different concentrations of RPV would affect HIV-1 detection and plasma viremia. Regardless of RPV concentrations at time of challenge, there was no significant delay in time to detect infection between untreated and mice challenged at days 1 ($p = 0.188$) or 7 after RPV LA dosing ($p = 0.144$) (Figure 11A). One animal challenged with WT HIV-1 had a blip in plasma viremia that approached $1x10^3$ vRNA copies/ml at week 7 post-challenge but was below the limit of detection at subsequent time points and was considered uninfected. Furthermore, plasma viremia was undetectable for two mice infected with WT HIV-1 at day 1 post-RPV LA dosing until week 7 post-challenge (Figure 11A). At week 5 post-challenge, the delay in detectable plasma viremia between untreated and day 1 post-RPV LA-treated groups approached significance ($p = 0.076$). Infected animals in both groups of RPV LA-treated mice had lower mean plasma viremia than untreated mice until week 5 post-challenge (Figure 12A), suggesting a transient suppressive effect on HIV-1 replication by RPV. Overall

plasma viremia levels were similar between the treated groups, indicating no advantage in virus suppression at higher concentrations of RPV. Plasma viremia levels in both RPV LA groups were not statistically different from untreated mice.

Table 10. Outcome of BLT mice treated with RPV LA and challenged with WT or drug-resistant HIV-1

Virus	Treatment[a]	Challenge route	Infected	p value[b]
WT	No Drug	vaginal	6/7	-
	RPV LA D1	vaginal	4/6[c]	0.559
	RPV LA D7	vaginal	4/6	0.559
Y181C	No Drug	vaginal	5/8	-
	RPV LA D1	vaginal	0/6	0.031
	RPV LA D7	vaginal	3/6[d]	0.999
Y181V	No Drug	vaginal	7/8	-
	RPV LA D1	vaginal	6/6	0.999
WT	No Drug	rectal	4/6	-
	RPV LA D7	rectal	2/5	0.567

[a] D1 and D7 represent days after RPV injection that mice were challenged with HIV-1.
[b] Results of Fischer's Exact Test for difference between No Drug and treatment groups of same virus and challenge route.
[c] A mouse in this group had detectable plasma viremia at one week and is considered uninfected.
[d] A mouse in this group had detectable plasma viremia but died before confirmation with a second sample and is considered uninfected.

Similar to mice challenged with WT HIV-1, there was no significant difference between the numbers of infected mice in the untreated and day 7 post-RPV LA-treated groups ($p = 0.999$) challenged with Y181C HIV-1 (Table 10). Five of 8 (62.5%) and 3/6 (50%) animals were infected in the untreated and treated groups, respectively. One mouse in the treated group had detectable plasma viremia at week 7 post-challenge, but died before infection could be confirmed with a follow-up sample and was considered uninfected. Time to detection of HIV-1 was not significantly different between these groups ($p = 0.509$) (Figure 11B), nor was the difference in mean plasma viremia levels ($p = 0.073$) (Figure 12B). The treated group had similar but lower

mean plasma viremia kinetics than untreated mice for the entirety of the study. However, all mice challenged at day 1 post-RPV LA-dosing were protected from Y181C HIV-1 transmission ($p = 0.031$) (Figure 11B), demonstrating that RPV LA can inhibit vaginal transmission of a low-level RPV-resistant virus.

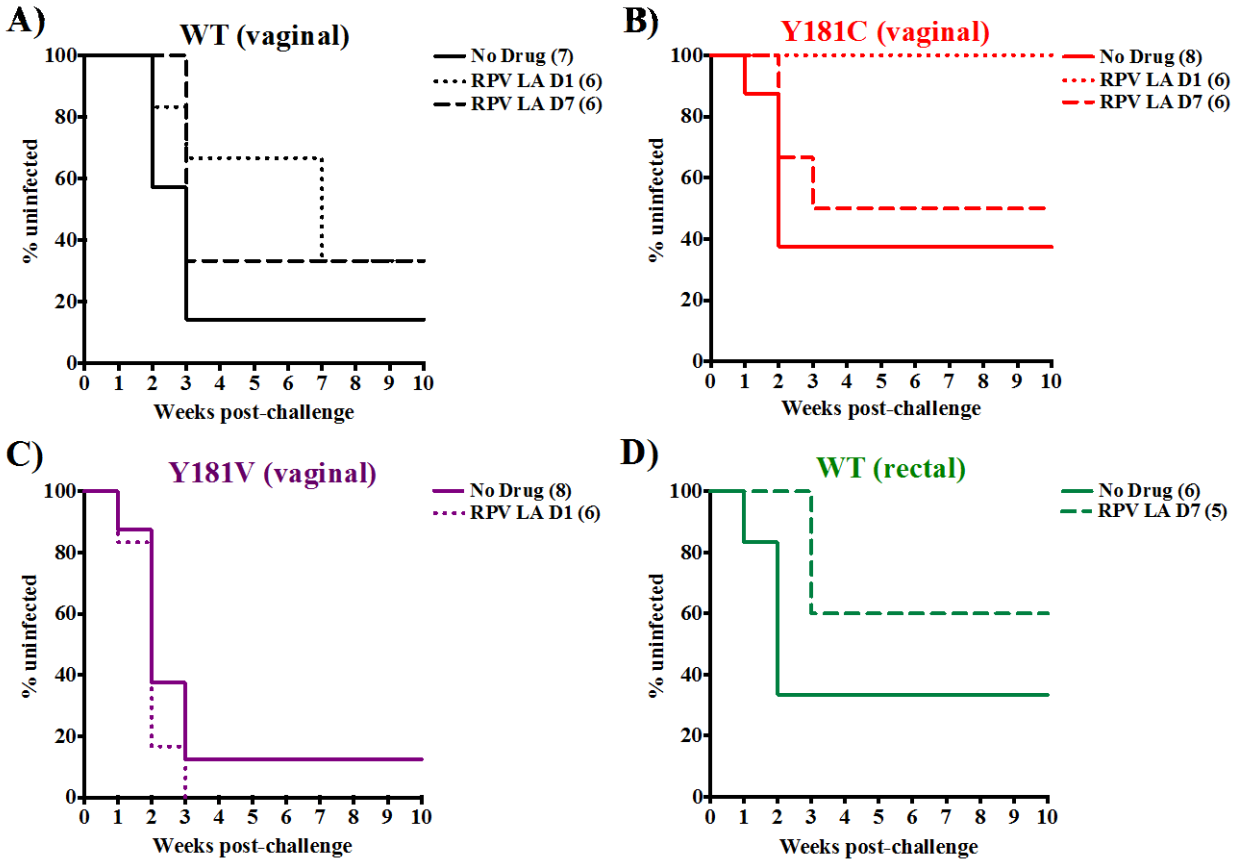

Figure 11. Time to detection of HIV-1 infection in BLT mice.
Kaplan-Meier plots display the number of BLT mice considered uninfected by HIV-1 at each week post-challenge as measured by qPCR (limit of detection of 400 vRNA copies/ml). Mice in each group were vaginally challenged (A-C) with 1 x 10^5 IU of WT (A, black), Y181C (B, red), or Y181V (C, purple) HIV-1 at 1 or 7 days post-RPV LA dosing. Another group of mice was rectally challenged (D) with 1 x 10^5 IU of WT (D, green) HIV-1. The number of mice per group is shown in parentheses.

As higher resistance is conferred by Y181V, only mice treated with RPV LA one day before virus inoculation were challenged with Y181V HIV-1. Infection rates were nearly identical in untreated and treated groups with 7/8 (87.5%) and 6/6 (100%) mice infected with

Y181V HIV-1, respectively. The difference in numbers of infected animals was not significant ($p = 0.999$) (Table 10). Similar to the other HIV-1 clones, there was not a significant difference in time to detect infection ($p = 0.361$) (Figure 11C). Unlike the mice infected by WT and Y181C viruses, mice infected with Y181V HIV-1 had nearly identical mean plasma viremia levels at all time points (Figure 12C), suggesting that RPV LA had no effect on Y181V HIV-1 transmission or replication post-infection.

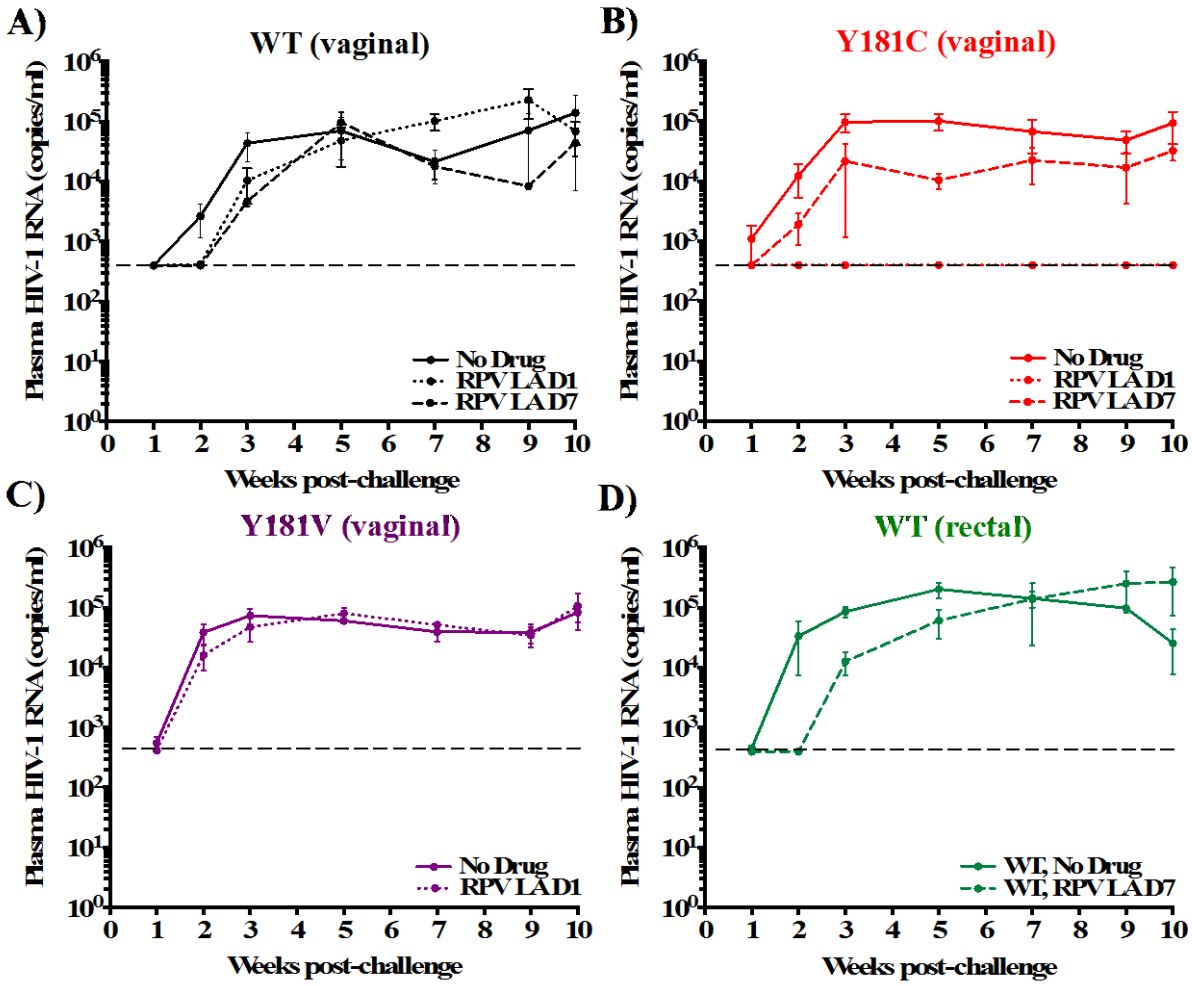

Figure 12. Plasma viremia in BLT mice infected with HIV-1.
Average and standard error mean of plasma viremia in untreated and RPV LA-treated BLT mice infected vaginally with WT (A, black), Y181C (B, red), or Y181V (C, purple) HIV-1 or infected rectally with WT (D, green) HIV-1. The black long-dash line represents the assay limit of detection (400 RNA copies/ml).

4.5.4 Rectal WT HIV-1 challenge of RPV LA-treated mice

A recent study reported that less RPV is required to inhibit HIV-1 infection of human colonic tissues compared to ectocervical explant tissues (349). Additional explant studies concluded that rectal tissues sampled from RPV LA-treated humans (600 or 1,200 mg doses) are protected from *ex vivo* HIV-1 infection if tissue RPV concentrations were ≥91 ng/ml, but protective values for cervical and vaginal tissues could not be calculated (348). To determine if less RPV LA is required to inhibit rectal HIV-1 transmission, BLT mice were rectally challenged with WT HIV-1 at day 7 post-RPV LA dosing. Two of 5 (40%) mice receiving RPV LA became infected with WT HIV-1, while 4/6 (66%) of the untreated animals became infected (Table 10). This difference was not statistically significant ($p = 0.567$), nor was there significant difference in time to detect HIV-1 between untreated and treated groups ($p = 0.201$) (Figure 11D). Similar to vaginal infections, the plasma viral load was initially transiently lower in the animals infected by rectal challenge; however, the mean plasma viremia was higher for untreated mice infected rectally compared to untreated mice infected vaginally (Figure 12A, D). Mean plasma viremia in the day 7 post-RPV LA rectal and day 1 post-RPV LA vaginal groups were nearly identical through week 9 post-challenge (Figure 12A, D). This result suggests that less RPV is required in rectal tissue to achieve the same effect in vaginal tissue, similar to results in Dezzutti *et al*. (349).

4.5.5 Sequencing for detection of RPV resistance mutations in treated mice

HIV-1 infection in people treated with a single antiretroviral drug can rapidly select DRMs (268-270), which was also observed in one participant in a RPV LA clinical trial (351). To determine if HIV-1 in PrEP-treated mice developed RPV-associated DRMs, virus isolated from the final

plasma sample from a subset of mice infected with WT or Y181C HIV-1 was sequenced. In the absence of RPV, no mutations were selected in WT virus, and Y181C was preserved (Table 11). In the presence of RPV, no DRMs were detected in RPV LA-treated mice infected with WT (n = 2), and Y181C (n = 3) was preserved with no additional DRMs. One silent base change and one codon change (G550K) in the RNaseH domain was detected in all sequences regardless of treatment or challenge virus (data not shown). G550K is not associated with resistance and is the consensus amino acid at this position according to the HIV Drug Resistance Database (266), thus G550K likely improves replication of our HIV-1 clone *in vivo*.

Table 11. Sequences of the RT coding region in untreated and RPV LA-treated mice infected with WT or Y181C HIV-1 as performed by population sequencing

Challenge virus	Animal	Treatment[a]	Challenge route	Weeks post-challenge	Sequence[b]
WT	448-004	No Drug	vaginal	10	WT
	943-005	No Drug	vaginal	10	WT
	943-004	RPV LA D7	vaginal	7	WT
	912-014	RPV LA D7	vaginal	10	A304V
Y181C	448-024	No Drug	vaginal	10	Y181C
	912-007	No Drug	vaginal	10	Y181C
	943-003	RPV LA D7	vaginal	10	E6K, Y181C
	912-009	RPV LA D7	vaginal	10	Y181C
	912-017	RPV LA D7	vaginal	10	Y181C

[a] Mice were either untreated or received RPV LA 7 days pre-challenge.
[b] Results represent the full RT coding region from the HIV-1 plasma population.

Because population-based sequencing detects mutations at a prevalence >20% of the population, we also performed single-genome sequencing on mice infected after treatment to detect mutations at a frequency of 2%. Of the 23 plasma samples subjected to single-genome sequencing, DRMs were detected in 11 of the animals within the first 277 bases of RT (Table 12). DRMs were detected in 2/5 (40%) of animals that did not receive RPV LA and 9/18 (50%) of animals that were treated. DRMs were not prevalent in more than 10% of the genomes

isolated per animal, thus population sequencing would have missed these mutations. We detected the NNRTI-associated resistance mutations K101E (linked to E138K in one animal), E138K/Q, Y188H, H221Y, P225H, and the NRTI-associated mutations L74V (linked to E138K in one animal) and M184I/V. The most prevalent DRM was the RPV-associated mutation E138K, which was detected in 2-9.5% of samples in 6 animals. H221Y was the only other mutation identified in more than one animal. Of note, the Y181C/V mutations remained constant as seen for Y181C with population sequencing. Only a single genome in two animals infected with Y181C HIV-1 displayed a reversion to Y181.

Samples from 8 animals were subjected to both sequencing methods. The A304V and E6K mutations identified by population sequencing (Table 11) in animals 912-014 and 943-003 were not found in any of the single genomes. This discrepancy may be attributed to differences in PCR or sequencing reaction conditions. Conversely, animals 943-004, 448-024, and 912-017 had one DRM in at least one sequence (Table 12), which were not detected by population sequencing.

Table 12. Detection of DRMs by single-genome sequencing in HIV-1 isolated from plasma of BLT mice

Challenge virus	Animal	Treatment[a]	Challenge route	Weeks post-challenge	DRMs (% of sequences)[b]
WT	448-004	No Drug	vaginal	14	n/a
	255-002	RPV LA D1	vaginal	10	n/a
	255-007	RPV LA D1	vaginal	10	n/a
	255-022	RPV LA D1	vaginal	10	H221Y (2.1)
	255-044	RPV LA D1	vaginal	8	E138K (6.4) K101E/E138K (2.1)
	912-008	RPV LA D7	vaginal	10	n/a
	912-014	RPV LA D7	vaginal	10	n/a
	943-004	RPV LA D7	vaginal	7	E138Q (2.2)
	255-012	RPV LA D7	rectal	10	n/a
	255-017	RPV LA D7	rectal	10	E138K (2.3) M184V (2.3)
Y181C	448-024	No Drug	vaginal	14	H221Y (2.2)
	912-007	No Drug	vaginal	10	n/a
	912-009	RPV LA D7	vaginal	10	n/a
	912-017	RPV LA D7	vaginal	10	M184I (4.3)
	943-003	RPV LA D7	vaginal	10	n/a
Y181V	255-031	No Drug	vaginal	10	E138K (2.4) L74V/E138K (4.5) L114V/E138K/G196R (2.4)
	943-021	No Drug	vaginal	10	n/a
	255-006	RPV LA D1	vaginal	5	Y188H (2.2)
	255-013	RPV LA D1	vaginal	10	n/a
	255-025	RPV LA D1	vaginal	10	E138K/G155E (2.2)
	255-038	RPV LA D1	vaginal	10	E138K (2.3) P225H (2.3)
	255-045	RPV LA D1	vaginal	10	E28K/E138K (2.3) E138K/D237N/D250N (2.3)
	255-056	RPV LA D1	vaginal	10	n/a

[a] Mice were either untreated or received RPV LA at 1 or 7 days pre-challenge.
[b] Results represent amino acid positions 1-277 of RT

4.6 DISCUSSION

Because of its safety, potency, and sustained drug release, RPV LA is under consideration as potential PrEP to inhibit HIV-1 infection; however, the global presence of NNRTI-resistant HIV-1 calls into question the utility of RPV LA PrEP to prevent transmission of virus with DRMs. High plasma concentrations of RPV LA were previously shown to inhibit vaginal transmission of WT HIV-1 clones in a BLT mouse model (501), but inhibition of WT and drug-resistant HIV-1 infection at biologically relevant concentrations of RPV has not been tested. Thus, BLT mice were treated with RPV LA and challenged with WT HIV-1 or clones with a single DRM that conferred low- (Y181C) or high-levels (Y181V) of RPV resistance. The primary goal of this work was to determine if RPV LA could inhibit mucosal transmission of WT and drug-resistant HIV-1. Secondarily, if RPV LA failed to prevent HIV-1 transmission, we also wanted to determine if breakthrough infection selects for drug resistance.

We found that RPV LA at plasma and mucosal tissue concentrations comparable to values reported in humans did not significantly inhibit vaginal transmission of either WT or Y181C HIV-1. As we had determined our stock of RPV LA to be effective, the lack of protection was attributed to inadequate RPV concentrations *in vivo*. Therefore, we performed a second round of vaginal challenges when RPV concentrations were approximately 8-fold higher in plasma and vaginal tissues and similar to plasma concentrations reported in a study that reported protection in RPV LA-treated mice challenged with transmitted/founder infectious molecular clones of HIV-1 (501). We found that the higher RPV concentrations did not significantly protect mice from a WT HIV-1 clone with robust replication, thus future experiments with clinical transmission/founder HIV-1 are planned. While our clone may be too robust for this model and clinical isolates are representative of actual circulating HIV-1,

sequence variability would introduce a confounding factor and would not allow a direct comparison of infection and prophylaxis between WT HIV-1 and clones with a single DRM.

Despite a lack of significant inhibition of WT HIV-1 transmission, mice challenged with Y181C HIV-1 were fully protected from infection at high RPV concentrations. This result shows that RPV LA can be protective in the event of drug-resistant HIV-1 exposure; however, our results also indicate that biologically relevant concentrations of RPV reported in humans (338) and recapitulated in our mice do not appear to be sufficiently protective. Like all NNRTIs, RPV binds highly to serum proteins (265, 556), and there is not a method of easily differentiating between protein-bound and free active RPV. Thus, the higher RPV concentrations for the mice challenged at day 1 post-RPV LA dosing may have provided enough free drug to inhibit Y181C HIV-1 and compensate for resistance provided by the mutation. While the 12.1 ng/ml $PAEC_{90}$ may be the protective threshold, current methods of RPV detection likely underestimate the concentration of active drug. Thus, higher RPV LA doses may be necessary to provide inhibitive concentrations; however, the feasibility and toxicity of such doses should be studied.

However, it could be argued the inhibition of Y181C HIV-1 at higher RPV concentrations could be attributed to viral fitness. Macaque studies have shown that infection rates by TFV- (K65R) or FTC-resistant (M1814V) SHIV clones are lower than WT SHIV in the presence of Truvada PrEP due to reduced fitness and not necessarily the presence of drug (440, 492). A reduction in mucosal transmission of K65R HIV-1 was also reported in humanized mice (509). However, results from our *in vitro* replication kinetics assay and a previous study (555) show that Y181C does not confer a substantial replication defect to HIV-1 when compared to WT virus. Also, similar numbers of untreated and sub-optimally treated animals were infected with our stock of virus; therefore, we credit the lack of Y181C HIV-1 infection to RPV activity.

While Y181C HIV-1 transmission was fully inhibited by RPV LA, the opposite was noted for Y181V HIV-1, which was expected given the level of resistance provided by the mutation (~30-fold). The lack of substantial difference in viral replication between WT and Y181V HIV-1, and its ability to break through RPV LA PrEP suggests that Y181V could be of greater clinical concern because its prevalence could increase if RPV LA is approved for PrEP. Y181V is already detected in treatment-experienced populations (557, 558) and is considered a surveillance transmitted DRM (309); thus, RPV LA users could be exposed to HIV-1 with the mutation and potentially become infected due to lack of prophylactic efficacy. Theoretically there is a concentration of RPV that should inhibit Y181V HIV-1 transmission, but the concentration of such a dose may not be tolerable or achievable *in vivo*.

Although we did not observe significant inhibition of WT HIV-1 transmission in RPV LA-treated animals, the time to HIV-1 detection results are interesting in regard to virus replication and resistance selection. While the same numbers of mice were infected in both RPV LA-treated groups challenged with WT HIV-1 (days 1 or 7 post-RPV LA), detection of HIV-1 was delayed until week 7 post-challenge in two mice challenged at day 1 after RPV LA dosing. This was also observed in one Y181C animal challenged at day 7 post-RPV LA injection. Therefore, RPV LA likely provided a therapeutic advantage by suppressing systemic viremia below the limit of detection for a sustained amount of time. We postulate that RPV waned after week 5 post-challenge to a concentration below which virus replication was no longer inhibited in the vaginal tissue. It is possible that a more sensitive vRNA detection method could have uncovered infection sooner, but the limitation in blood draw volumes in mice does not allow for high sensitivity of vRNA detection and greater sampling frequency. Nonetheless, our results raise the following questions: would WT HIV-1-infected animals maintain undetectable levels of

plasma viremia over the course of monthly RPV LA doses, or would low-level undetectable replication eventually select for drug-resistant variants? While we did not detect any DRMs by population sequencing in a subset of animals infected with WT or Y181C virus at 7 days post-RPV LA dosing, single-genome sequencing of a broader subset of infected mice revealed the selection of DRMs in nearly half the animals, albeit at <10% of the individual virus populations. Interestingly, DRMs not associated with RPV were selected, thus displaying how stochastic the HIV-1 mutation process can be. These results recapitulate the sequencing results seen in our macaques given RPV LA monotherapy (Chapter 3); however, sequencing of other time points could be performed to determine the stability of the DRMs and if RPV truly selects for resistance or if the detected DRMs were transient random events from mutational bias or hypermutations (546, 547). The idea of a "smoldering" HIV-1 infection presents the opportunity to use reporter viruses and visual techniques, such as *in vivo* bioluminescence or immunofluorescence assays, to identify sanctuary sites where virus replication occurs despite systemic drug concentrations. We could then study drug penetration at sanctuary sites through PK analysis and track HIV-1 evolution under near-suppressive drug pressure using sensitive sequencing techniques. Such studies could reveal areas of improvement for RPV LA dosing and better identify the circumstances of DRM evolution.

While the majority of new HIV-1 infections occur through vaginal exposure, rectal tissue is also a significant route of transmission (10). RPV LA is shown to penetrate both tissue compartments and concentrate to levels that should be protective (338, 348). However, human explant models indicate that there is a difference in RPV protection between the two mucosal tissue compartments, which is likely due to differences in RPV penetration (348, 349). Our results were unclear due to statistical power, but they suggest that RPV LA may be more

effective at inhibiting HIV-1 transmission in rectal rather than vaginal tissue. Through week 5 post-challenge, the numbers of infected animals were similar between vaginally and rectally challenged groups at days 1 and 7 post-RPV LA injection, respectively. Had the number of infected animals remained the same through the end of the study, our data would have mirrored those of Dezzutti *et al*. where higher RPV concentrations would be required to be equally protective in ectocervical tissue as colonic tissue (349). Elucidating the difference in the ability of RPV LA to inhibit HIV-1 transmission by mucosal compartment will be important as RPV LA trials are currently focused on women at risk of vaginal infection; whereas, our results and published explant data indicate that RPV LA may be more suitable for populations at risk of rectal infection.

In conclusion, our data suggest that RPV LA at biologically relevant concentrations reported in humans is ineffective at significantly inhibiting vaginal transmission of WT or drug-resistant HIV-1. However, we do show that RPV LA can inhibit vaginal transmission of HIV-1 with a low-level RPV resistance mutation at plasma and tissue concentrations nearly 1-log higher than those reported in humans. We also showed that virus with high RPV resistance can be transmitted in the presence of high concentrations of RPV, which may not be achievable or sustained *in vivo*. Thus, we conclude that the level of resistance conferred by a mutation can affect PrEP efficacy. Our data also support that selection of DRMs after breakthrough infection during RPV LA PrEP may be difficult as RPV-associated DRMs were selected but only in a small number of animals and at low prevalence. Overall, our study raises more questions with RPV LA that should be addressed to better understand the effect of HIV-1 resistance on the PrEP strategy. Also, careful consideration, specifically in regard to dosing, should be made before moving RPV LA into Phase III clinical trials to test the efficacy of this PrEP modality.

5.0 AIM 3: A RILPIVIRINE ANALOG SELECTS FOR A NOVEL COMBINATION OF DRUG-RESISTANT MUTATIONS IN HIV-1 REVERSE TRANSCRIPTASE

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5.1 PREFACE

Results from this research were presented as a poster titled "*In vitro* HIV-1 infection in the presence of a rilpivirine analog selects for a novel combination resistance mutation" at the 2015 Palm Springs Symposium on HIV/AIDS in Palm Springs, California.

Results from this work were included in two publications:

Our results were included in two publications:

1) Smith SJ, Pauly GT, Akram A, Melody K, Ambrose Z, Schneider JP, Hughes SH. 2016. Rilpivirine and doravirine have complementary efficacies against NNRTI-resistant HIV-1 mutants. *J Acquir Immune Defic Syndr* 72: 485-491.

2) Smith SJ, Pauly GT, Akram A, Melody K, Rai G, Maloney DJ, Ambrose Z, Thomas CJ, Schneider JT, Hughes SH. 2016. Rilpivirine analogs potently inhibit drug-resistant HIV-1 mutants. *Retrovirology* 13: 11.

The work presented in this chapter is for completion of Aim 3 in partial fulfillment of the requirements for the degree of Doctor of Philosophy. Kevin Melody performed all work reported in this chapter.

5.2 ABSTRACT

Analogs of the FDA-approved NNRTI RPV were designed to preserve the potency and tolerability of RPV and broaden efficacy against drug-resistant HIV-1. Three analogs, 7, 11, and 13, showed similar *in vitro* efficacy against a panel of NNRTI-resistant HIV-1 isolates and were chosen for resistance selection to understand the mechanisms of HIV-1 antiretroviral activity. Resistance selection was conducted by passaging HIV-1-infected cells in increasing concentrations of the compounds. The RT coding region of virus isolated from culture supernatants was sequenced and analyzed for mutations that could confer drug resistance. Compounds 7 and 11 selected for known RPV-associated resistance mutations K101E, E138K, Y181C, or M230I in single clones, which showed similar resistance phenotypes to the analogs as to RPV. However, compound 13 consistently selected a combination of two mutations in RT: G112D and M230I. Virus with either G112D or M230I alone conferred resistance to the NRTI FTC or all NNRTIs, respectively. Together, G112D and M230I conferred a >2-fold decrease in NNRTI susceptibility beyond that conferred by M230I alone, but the presence of M230I abrogated FTC resistance of G112D and caused hypersusceptibility to three NRTIs and a pyrophosphate analog. Replication assays revealed that G112D/M230I virus has better replication kinetics than virus with either single mutation, yet all three mutants displayed decreased replication capacity compared to WT HIV-1. Our data suggests that RPV analog 13 selects a combination of mutations through a novel mechanism of action. RT polymerization assays and modeling of drug binding is underway to confirm the results of our drug susceptibility assays and to better understand the resistance conferred by G112D, M230I, and G112D/M230I.

5.3 INTRODUCTION

Due to lack of fidelity by RT during reverse transcription (239, 240) and a high replication rate (263), HIV-1 evolves frequent mutations. These mutations can lead to the selection of isolates with DRMs that are not fully suppressed by therapeutic agents (264). Thus, the evolution of drug-resistant HIV-1 necessitates adjusting ART regimens to maintain viremia suppression in HIV-1^+ individuals. Some DRMs can confer cross-resistance to all drugs of the same class (16). Consequently, evolution of cross-resistant DRMs could eliminate the use of an entire antiretroviral drug class, thus limiting alternative ART regimens in HIV-1^+ individuals.

Due to increasing prevalence of HIV-1 drug resistance (285), new antiretroviral drugs effective against viruses with known DRMs are needed. RPV is a diarylpyrimidine NNRTI that possesses flexible arms, which allows it to better conform to changes in RT structure and maintain efficacy against common DRMs associated with more rigid NNRTIs (265, 286, 288). In *in vitro* studies, RPV was shown to have a higher genetic barrier to resistance than the NNRTIs EFV and NVP (265). While the use of EFV and NVP is often abrogated by a single non-synonymous base change that leads to high levels of resistance, RPV-associated mutations with single base changes confer low-level reductions in susceptibility (<4-fold change). High-level resistance (>10-fold change) is conferred by 2 base changes in a single codon (*e.g.*, Y181I/V) or multiple amino acid mutations (*e.g.*, K103N/Y181I) (265, 303). Compared to EFV, RPV was shown in two phase III clinical trials to have similar efficacy, better tolerability, and selection of different DRMs (292). Participants who failed treatment predominantly selected for the DRMs K103N or E138K in the EFV or RPV arms, respectively (306). Efficacy against common NNRTI-resistant mutations, a higher genetic barrier to resistance, and a favorable safety profile makes RPV an attractive NNRTI; however, the mechanism of action of RPV to prevent HIV-1

replication is similar to all approved NNRTIs, and cross-resistant DRMs exist that would negate the use of this drug class (49, 218, 265, 288)

To address cross-resistance in NNRTIs, analogs of RPV with changes to the pyrimidine and 4-amino-benzonitrile moieties were developed to maintain the potency and safety of RPV while potentially altering the mechanism of action (543). The acrylonitrile ring moiety was left unchanged because it is essential for RPV function as it binds a hydrophobic pocket in RT (286, 288, 289). Of 22 analogs tested, three compounds displayed similar therapeutic indices and antiretroviral efficacies as RPV in a panel of HIV-1 with mutations in RT including L100I, K103N, V106A, E138K, Y181C, Y188L, H221Y, and K103N/Y181C (543). It is possible that the modifications made in these analogs result in new mechanisms of action that would select for novel resistance mutations. If this is the case, these analogs could reveal new virus-drug interactions that could enhance our understanding of HIV-1 biology and lead to development of new RT inhibitors for use in ART or PrEP. The goal of this work was to investigate the genotypic and phenotypic resistance profiles of a subset of these RPV analogs: 7, 11, and 13 (Figure 13). By determining the resistance profile of these compounds, we can infer their mechanisms of HIV-1 inhibition. We hypothesized that the analogs would select for DRMs that would not display cross-resistance to other NNRTIs.

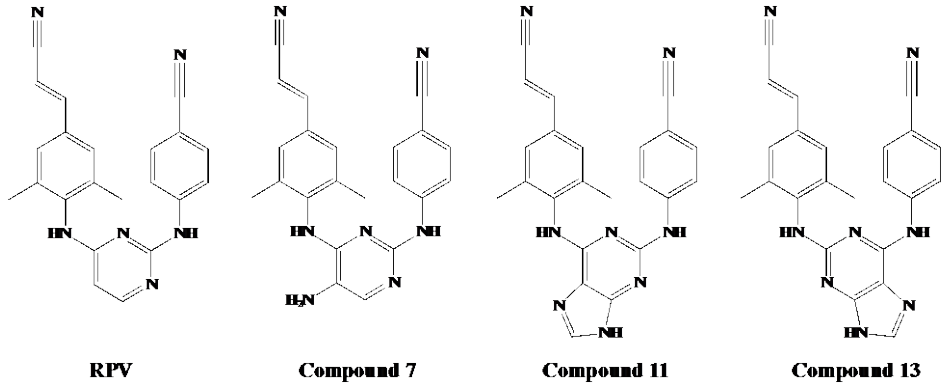

Figure 13. Chemical structures of RPV and analogs 7, 11, and 13.

5.4 MATERIALS AND METHODS

5.4.1 Cell lines and compounds

293T, TZM-bl, and GHOST-R3/X4/R5 were maintained as described in section 3.41. HuT-R5 (559) cells were grown in RPMI 1640 media (Invitrogen) supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.292 mg/ml L-glutamine, 500 μg/ml G418, and 0.5 μg/ml puromycin. All cell lines and cell culture experiments were maintained at 37º C with an atmosphere of 5% CO_2.

RPV and RPV analogs 7, 11, and 13 were kindly provided by Steve Hughes (543). NVP, EFV, ETR, ddI, FTC, TFV, and AZT were obtained from the AIDS Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH. Foscarnet (PFA) was a kind gift provided by Dr. John Mellors.

5.4.2 HIV-1 production

HIV-1_{xxLAI}, a HIV-1_{LAI} clone with silent restriction sites within RT (534), was used in all cell culture assays and will hereafter be referred to as HIV-1. RT mutations were made in the proviral plasmid using the QuikChange II XL site-directed mutagenesis kit (Agilent Technologies). Viruses were generated by transfection of 293T cells and titered on GHOST-R3/X4/R5 cells as described in section 3.4.2.

5.4.3 Selection of HIV-1 resistance mutations with RT inhibitors

HuT-R5 cells were infected with HIV-1 at a MOI of 0.05 for 2 hours at 37º C. After virus exposure, cells were cultured in media containing inhibitors at a concentration 4-fold above the reported EC_{50} (543). Cell cultures were split every 2-3 days until CPE was noted. After observation of CPE, aliquots of cell-free supernatant were harvested and stored at -80º C and drug concentrations in the cultures were increased 2-fold.

vRNA was isolated from cell-free supernatants with the RNeasy Mini Kit (Qiagen). cDNA was generated from recovered vRNA using the SuperScript III First-Strand Synthesis kit using random hexamer primers (Invitrogen). vDNA was isolated from supernatant-free infected cells by the QiAmp DNA Mini Kit (Qiagen). The RT coding region was amplified using Platinum PCR Supermix (Invitrogen) with primers LAI-RTF (5'-TTTGCCAGGAAGATGGAAAC-3') and LAI-R (5'-TCACTAGCCATTGCTCTCCA-3'). PCR reaction conditions were 94º C for 2 min; 35 cycles of 94º C for 30 s, 52º C for 30 s, 72º C for 2 m; and one cycle at 72º C for 5 min. Amplified PCR products were cloned into TOPO vectors using a pCR 2.1-TOPO TA Cloning kit (Invitrogen). Bacteria colonies were screened for full-length RT insertion by PCR with the aforementioned primers and reaction conditions. Colonies positive for RT were cultured overnight at 37ºC in Luria-Bertani broth (Mo Bio Laboratories, Inc.) supplemented with 50 μg/ml carbenicillin (Thermo Fischer Scientific). Plasmid was recovered using the QIAprep Spin Miniprep Kit (Qiagen) and sequenced using primers LAI-RT-F, 200F, and 350F. Sequences were analyzed using Lasergene (DNASTAR).

5.4.4 Drug susceptibility assays

Susceptibility to RPV analogs, NNRTIs, NRTIs, and a pyrophosphate analog were performed as described in section 4.4.3.

5.4.5 HIV-1 replication assay

Virus replication was measured as described in 4.4.4 with the use of HuT-R5 cells and not PBMCs. A Retrotek HIV-1 p24 antigen ELISA kit (ZeptoMetrix) was used for the data displayed in Figure 15.

5.5 RESULTS

5.5.1 Selection of DRMs in HIV-1 by RPV and compounds 7 and 11

To select mutations in HIV-1 that confer resistance to the RPV analogs, HuT-R5 cells were infected with WT HIV-1 and passaged in increasing concentrations of RPV or analogs until viral replication was similar in the absence of drug. The RT coding region of vRNA isolated from culture supernatants was amplified by PCR, cloned, and sequenced. After each concentration change, RPV and compounds 7 and 11 displayed similar CPE patterns of large syncytia and substantial cell death and were increased gradually to concentrations 128-fold above their respective EC_{50} values until virus replication was robust within 60 days post-infection (Figure 14A). Sequencing of RT clones from RPV, compound 7, and compound 11 cultures revealed the

selection of RPV-associated mutations at ≥64-fold the starting EC_{50} values (Table 12). K101E was detected in 43% of clones isolated from the RPV culture, and this mutation is a known RPV-associated DRM (16, 265, 306). Like RPV, compound 7 selected K101E, albeit in 100% of clones (Table 12).

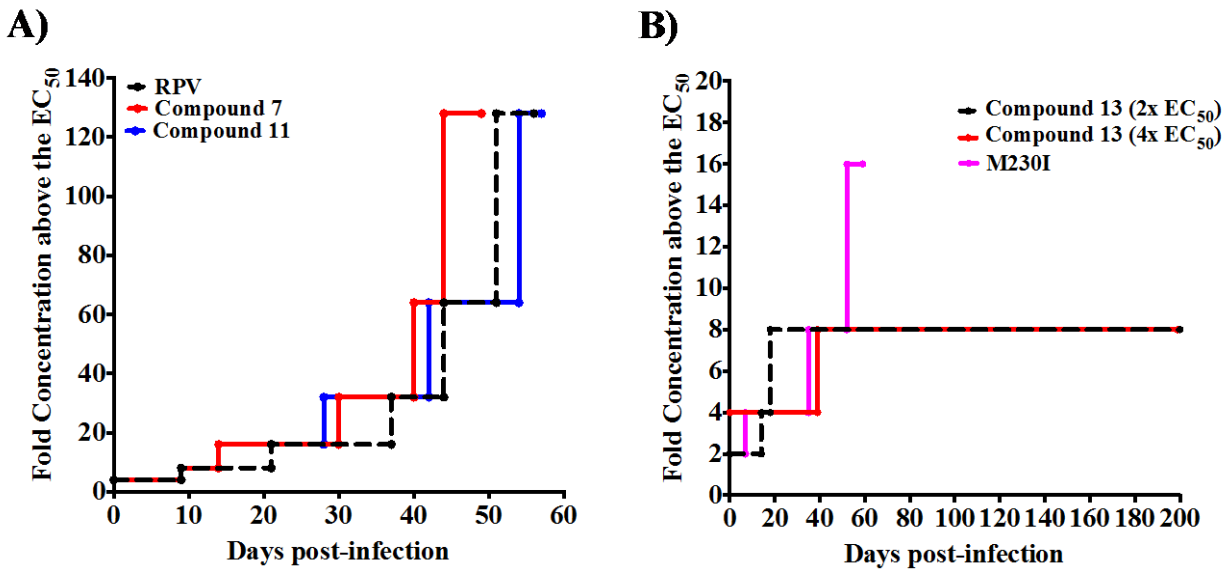

Figure 14. Increase in RT inhibitor concentrations during HIV-1 resistance selection. HuT-R5 cells infected with HIV-1 and cultured in the presence of RPV or RPV analogs were monitored for CPE, at which time RT inhibitor concentrations were increased. Resistance selection in WT HIV-1-infected cells cultured in RPV, compound 7, or compound 11 were started at 4-fold above their respective EC_{50} (A). Alternatively, two resistance selection cultures of WT HIV-1 in compound 13 were started at 2- or 4-fold above the EC_{50}, while M230I HIV-1 selection in compound 13 was started at 4-fold above the EC_{50} (B).

Compound 11 selected a more complex resistance profile than compound 7. RPV-associated resistance mutations E138K, Y181C, and M230I were detected separately in 10%, 50%, and 20% of clones, respectively (Table 12). However, two mutations not previously associated with RPV resistance were discovered with consistency. E40K was detected in 50% of clones, both alone and with Y181C, indicating a possible linkage between the mutations. D67E was detected in 20% of clones along with Y181C or M230I. The lack of consistent DRMs

indicates that selection in compound 11 may not have been complete at termination of the cultures. Because both compounds 7 and 11 selected known RPV-associated DRMs, selection experiments were not repeated.

Table 13. RT sequences of HIV-1 clones isolated from supernatant of cell cultures in RPV, compound 7, and compound 11

Compound	Total days in selection	Fold-change from EC_{50}	Clone	Mutations in RT[a]
RPV	56	128	1-3	WT
			4-5	**K101E**
			6	S489P
			7	**K101E**, S489P
Analog 7	49	128	1-7	**K101E**
			8	**K101E**, S105I^{FS}
			9	**K101E**, I375*
			10	**K101E**, G456R
Analog 13	57	64	1	E40K
			2	L246P
			3	**E138K**
			4-5	E40K, V111A, **Y181C**
			6	D67E, **Y181C**, T400A
			7	E40K, **Y181C**, L301P
			8	D67E, S105I^{FS}, K154E^{FS}, **M230I**
			9	M16V, **M230I**, Q330*, K465E, G556R
			10	E40K, T69A, **Y181C**, L228P, I375N

[a] Bold, RPV-associated mutations.
FS, frameshift; *, stop codon.

5.5.2 Selection of a novel combination of RT mutations in HIV-1 by compound 13

Unlike selection with RPV and compounds 7 and 11, cells cultivated in the presence of compound 13 displayed few small syncytia and maintained large cell clusters similar to an uninfected culture. Because of the lack of robust CPE, concentrations of compound 13 were not increased as frequently or as high compared to RPV and compounds 7 and 11 (Figure 14B). CPE

suppression by compound 13 was maintained at a concentration 8-fold above the EC_{50} for >130 days in two separate cultures started at 2- or 4-fold above the EC_{50}.

vDNA from cells and vRNA from supernatant were isolated at day 156 post-infection from the culture begun at 2-fold the EC_{50}. The mutations G112D and M230I were identified in near full-length RT sequences from both vDNA and vRNA populations (Table 13). This mutation combination was also observed in 2/3 of vRNA clones we could obtain, but only M230I alongside K101E was detected in the single vDNA clone. Sequencing of population and clonal vDNA and vRNA from the culture started at 4-fold above the EC_{50} also revealed G112D and M230I linkage, which was observed in 100% of the sequences.

To determine if M230I would allow quicker selection of G112D in the presence of compound 13, HuT-R5 cells were infected with M230I HIV-1 and cultured in media containing compound 13. Seven days after switching the compound concentration to 16-fold above the EC_{50} (Figure 14B), G112D and K196R were detected by population sequencing of vRNA (Table 13). Interestingly, sequencing of additional archived supernatant samples from the culture started at 2-fold above the EC_{50} revealed that G112D evolved prior to M230I and was observed as early as 63 days post-infection (Table 13). This was similar to the time needed for G112D to evolve in M230I HIV-1 in the presence of compound 13. Our data suggest that G112D is not a spontaneous mutation and arises with M230I to provide an advantage to HIV-1 in the presence of RPV analog 13 *in vitro*.

5.5.3 Resistance phenotypes of RT mutants selected by RPV and RPV analogs

Viruses with known RPV-associated mutations were resistant to RPV and the respective compounds in which they were selected (Table 14). The mutations K101E, E138K, and Y181C

in HIV-1 conferred low-level resistance to RPV, which is comparable with previous reports (265, 543). Although only consistently detected in RPV and compound 7 cultures, K101E HIV-1 was tested for resistance against all three RPV analogs because it is a known DRM selected *in vitro* and *in vivo* by RPV (265, 283, 306) and was not included in the original analog experiment (543). K101E conferred low-level resistance to RPV and compounds 11 and 13 compared to WT HIV-1 (2.8- to 3.5-fold), but resistance was slightly higher against compound 7 (6.0-fold). This difference between K101E resistance to RPV and compound 7 was similar in another study (560). E138K and Y181C also conferred low-level resistance to both RPV and compound 11 (2.0- to 3.5-fold). However, virus with Y181C and E40K or D67E had similar resistance to RPV and compound 11 as Y181C alone (3.0- to 3.5-fold).

Of the known mutations tested, M230I HIV-1 showed the highest resistance to RPV and analogs (Table 14). The EC_{50} values of RPV and compounds 11 and 13 were 7.3- to 9.8-fold higher for M230I HIV-1 than WT HIV-1; however, EC_{50} values were similar for D67E/M230I HIV-1 and M230I HIV-1, suggesting that D67E does not have a direct role in resistance. The EC_{50} values for compound 11 to M230I HIV-1 and the culture supernatant were similar, implying that M230I is the mutation mainly responsible for HIV-1 resistance to compound 11 in the culture (Table 12). Of note, the addition of G112D to M230I tripled the resistance of M230I HIV-1 to both RPV (30.3-fold) and compound 13 (20.4-fold), whereas G112D alone was as susceptible as WT virus to both compounds (1.1- to 1.8-fold). The EC_{50} values of compound 13 to G112D/M230I HIV-1 and the compound 13 supernatant were similar, suggesting that the dual mutant is likely responsible for resistance in our compound 13 culture.

Table 14. RT sequences of HIV-1 populations and clones isolated from supernatant of cell cultures compound 13

Virus	Days of selection	Nucleic acid source	Positions sequenced	Mutations in RT[a]
WT	63	RNA (population)	1-250	K64*, S69C, G112D
(2x EC_{50} start)	91	RNA (population)	1-250	G112D, **M230I**
	156	RNA (population)	1-140, 228-450	G112D, **M230I**, V381L
		DNA (population)	1-120, 210-560	G112D, **M230I**
		RNA (clone)	1-560	G112D, **M230I**
		RNA (clone)	1-560	V10I, G112D, K126E, **M230I**
		RNA (clone)	1-560	K43R, E53G, K281*, E297G, A360V, P433L
		DNA (clone)	1-560	**K101E**, **M230I**, L391P
WT	199	RNA (population)	1-560	G112D, **M230I**
(4x EC_{50} start)		DNA (population)	1-560	G112D, **M230I**
		RNA (clone)	1-560	G112D, **M230I**
		RNA (clone)	1-560	G112D, **M230I**
		RNA (clone)	1-560	G112D, **M230I**, $L517F^{FS}$
		RNA (clone)	1-560	G112D, **M230I**, D250V, T403I
		DNA (clone)	1-560	G112D, **M230I**
		DNA (clone)	1-560	G112D, **M230I**, K331E
		DNA (clone)	1-560	P55L, G112D, **M230I**, R448G
M230I	59	RNA (population)	1-470	G112D, K196R, **M230I**

[a] Bold, RPV-associated mutations.
FS, frameshift; *, stop codon.

Table 15. *In vitro* antiviral activity of RPV and compounds 7, 11, and 13 against WT or mutant HIV-1

	EC_{50} (nM) (Fold-change above WT)[a]			
Virus	RPV	Compound 7	Compound 11	Compound 13
Supernatant[b]	-	23.6 (12.4)	5.5 (9.2)	24.8 (20.7)
WT	0.4 ± 0.1 (1.0)	1.9 ± 0.4 (1.0)	0.6 ± 0.1 (1.0)	1.2 ± 0.2 (1.0)
K101E	1.4 ± 0.4 (3.5)	11.4 (6.0)	1.7 ± 0.4 (2.8)	3.6 ± 0.9 (3.0)
G112D	0.7 ± 0.04 (1.8)	-	-	1.3 ± 0.3 (1.1)
E138K	1.0 ± 0.2 (2.5)	-	1.2 ± 0.3 (2.0)	-
Y181C	1.4 ± 0.3 (3.5)	-	1.7 ± 0.3 (2.8)	-
E40K/Y181C	1.4 ± 0.4 (3.5)	-	1.8 ± 0.3 (3.0)	-
D67E/Y181C	1.2 ± 0.2 (3.0)	-	1.7 ± 0.2 (2.8)	-
M230I	3.9 ± 0.6 (9.8)	-	5.9 ± 1.3 (9.8)	8.8 ± 1.3 (7.3)
D67E/M230I	3.4 ± 0.6 (8.5)	-	6.2 ± 1.8 (10.3)	-
G112D/M230I	12.1 ± 1.1 (30.3)	-	-	24.5 ± 1.9 (20.4)

[a] Average and standard deviation values based on 3-6 independent experiments each performed in triplicate. Bulk virus results based on 1 independent experiment performed in triplicate.
[b] Virus population in supernatant at time of DRM detection.
-, not done.

5.5.4 Effect of select RT mutations on HIV-1 replication

Since the D67E mutation did not enhance resistance, we hypothesized that it may act as a compensatory mutation to increase replication capacity of HIV-1 with the DRMs Y181C or M230I. To measure virus replication *in vitro*, HuT-R5 cells were infected with WT, M230I, or D67E/M230I HIV-1 and passaged for 12 days. Virus production was measured by HIV-1 capsid protein (p24) in the culture supernatants (Figure 15A). Cells infected with WT HIV-1 demonstrated high levels of CPE and cell death early, which explains the initial exponential increase in p24 production followed by a plateau after day 4 post-infection. In contrast, HIV-1 with M230I or D67E/M230I mutations produced approximately 1-log less p24 through day 4 and surpassed the WT culture after day 6 post-infection. Overall, the replication curves for M230I and D67E/M230I were similar suggesting that D67E is not a compensatory mutation. Replication assays were not performed for E40K viruses.

The consistent detection of G112D and M230I mutations by sequencing HIV-1 selected in compound 13 cultures suggests an advantage for virus with both mutations; although, G112D alone did not confer resistance to RPV or compound 13. Replication kinetics of WT, G112D, M230I, and G112D/M230I HIV-1 indicate that the single mutations cause reduced replication capacity (Figure 15B). G112D HIV-1 and M230I HIV-1 displayed a 1- to 2-log difference in p24 concentrations through day 6 of the assay and had similar growth curves. Similar to the D67E/M230I experiment, WT virus p24 production plateaued after day 6, whereas the single mutant viruses continued to produce p24 after 6 days post-infection. However, G112D appears to be compensatory to M230I as replication capacity was 0.5- to 1.0-log higher for the double versus single mutant viruses through day 6 post-infection. While not as efficient at p24

production as WT HIV-1 early in the experiment, the replication curve of G112D/M230I HIV-1 mirrored that of WT HIV-1 with a plateau in virus production after day 6 post-infection. Based on two separate replication assays using these viruses, replication fitness is ranked as WT > G112D/M230I > G112D > M230I. Combined with the resistance data, it appears that the G112D mutation is responsible for improved replication and increased resistance in M230I HIV-1.

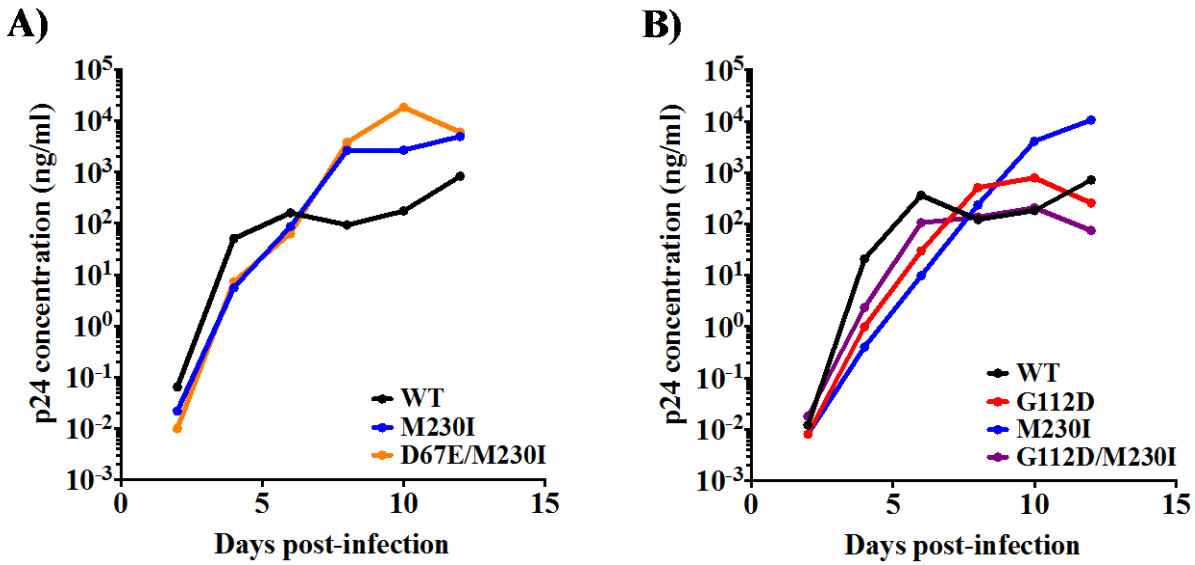

Figure 15. Replication kinetics of WT and mutant HIV-1.
HuT-R5 cells were infected with WT or mutant HIV-1, and p24 concentrations in culture supernatants were measured by ELISA. Concentrations of p24 in cultures infected with WT, M230I, or D67E/M230I HIV-1 (A) or WT, G112D, M230I, or G112D/M230I HIV-1 (B) are shown. Results of (B) are representative of two independent experiments.

5.5.5 G112D/M230I HIV-1 susceptibility to NNRTIs and NRTIs

Although G112D/M230I HIV-1 had enhanced resistance to RPV and compound 13 and improved replication capacity over virus with either mutation alone, we investigated the inhibition of this double mutant by a broader panel of RT inhibitors. Testing the mutant virus for susceptibility to other RT inhibitors will determine if there is a cross-resistance profile and

subsequently how the mutant may avoid inhibition by compound 13. WT, G112D, M230I, and G112D/M230I HIV-1 were tested for susceptibility against a panel of FDA-approved NNRTIs (EFV, ETR, and NVP), NRTIs (AZT, ddI, FTC, TFV), and a pyrophosphate inhibitor (PFA) (Table 15). Similar to RPV and compound 13, G112D HIV-1 had no resistance to other NNRTIs and the NRTI TFV (0.7- to 1.2-fold). However, this mutant did have intermediate resistance (16.1-fold) to FTC, while displaying hypersusceptibility to AZT and PFA (0.2-fold). Conversely, M230I conferred resistance to all three NNRTIs (6.5- to 10.6-fold) and hypersusceptibility to all NRTIs (0.1- to 0.3-fold). Virus with both G112D and M230I had increased resistance to NNRTIs compared to M230I alone (16.5- to 17.9-fold) as was seen with RPV and compound 13. Interestingly, the addition of M230I to G112D virus abrogated the resistance to FTC (3.9-fold) observed with G112D alone. The dual mutant also displayed hypersusceptibility to AZT (0.1-fold) and PFA (0.1-fold), and a mild increase in susceptibility to TFV (0.6-fold). Our results indicate that HIV-1 with G112D and M230I is cross-resistant to all clinically relevant NNRTIs and FTC, susceptible to ddI and TFV, but hypersusceptible to AZT and PFA.

Table 16. Change in *in vitro* susceptibility of RT inhibitors against G112D, M230I, or G112D/M230I HIV-1

	EC_{50} Fold-Change above WT[a]							
Virus	ETR	EFV	NVP	AZT	ddI	FTC	TFV	PFA
G112D	1.2	1.2	0.7	0.2	2.0	16.1	0.7	0.2
M230I	6.5	8.2	10.6	0.2	0.1	0.1	0.3	0.3
G112D/M230I	16.5	17.9	17.0	0.1	0.7	3.9	0.6	0.1

[a] Values represent fold-change based on mean mutant EC_{50}/ mean WT EC_{50}. All data are representative of 3 individual experiments, each performed in triplicate.

ETR, etravirine; EFV, efavirenz; NVP, nevirapine; AZT, zidovidine; ddI, didanosine; FTC, emtricitabine; TFV, tenofovir; PFA, foscarnet.

5.6 DISCUSSION

Despite the efficacy of available antiretroviral drugs to suppress HIV-1 replication, DRMs evolve in the virus and limit alternative therapy options, especially if the mutations impart cross-resistance to multiple drugs. To counter drug resistance, new compounds must be developed that are potent, safe, and effective against isolates with known resistance mutations. The goal of this work was to determine the resistance profile of three RPV analogs that met these criteria (543), which would shed light on their mechanisms of action as HIV-1 inhibitors. Because of the structural changes to RPV that would affect drug-virus interactions, we hypothesized that these analogs could select novel resistance mutations in HIV-1 RT.

Results from our *in vitro* assays demonstrate that compounds 7 and 11 select for known RPV-resistant RT mutations K101E, E138K, Y181C, and M230I. These mutations were previously selected under RPV pressure *in vitro* and in clinical trials (265, 283, 295, 306). Detection of K101E HIV-1 after selection with compound 7 is not surprising because modeling shows this compound forms a H-bond between its pyrimidine moiety and the K101 residue of RT (543), similar to RPV (288). Loss of this interaction through K101E likely reduces the binding capacity of compound 7; however, the decrease in susceptibility conferred by K101E did not account for the total resistance seen in our culture supernatant. Genotypic evidence indicates that K101E is a key mutation in HIV-1 resistance to compound 7, but other undetected mutations may account for the greater resistance seen in the virus supernatant. Additional selection cultures could be performed to address this disparity.

In contrast to selection of a single HIV-1 DRM by compound 7, resistance to compound 11 was more complex. Three distinct RPV-associated DRMs, E138K, Y181C, and M230I, were detected in vRNA isolated from the compound 11 culture. The selection of these DRMs by compound 11 is likely attributable to its proposed binding to RT, which is similar to RPV (560). The change from a pyrimidine (RPV) to a purine (compound 11) moiety allows the central ring to sit deeper in the NNRTI-binding pocket while maintaining hydrogen bonds with K101 and the shared water molecule between the drug and E138 (560), indicating that K101E also causes resistance to compound 11. The cyanovinyl and benzonitrile moieties of compound 11 occupy a similar confirmation as RPV in RT (560), which could explain the similar resistance Y181C and M230I to both RPV and compound 11. Additional mutations not associated with NNRTI resistance, E40K and D67E were also detected in combination with Y181C or Y181C and M230I, respectively. The selection of E40K with Y181C and/or M230I was previously reported in RPV and ETR selection cultures (265, 548). Similarly, D67E has been observed in ART-treated patients with NRTI-associated resistance mutations (561, 562), but it was not associated with phenotypic resistance (561). However, these mutations did not meaningfully alter the resistance phenotype of Y181C or M230I to RPV or compound 11, and D67E did not appear to provide a compensatory benefit to M230I HIV-1 replication capacity. A study by our collaborators revealed that D67E, but not E40K, confers low-level resistance to RPV and compounds 7 and 13, while neither mutation conferred resistance to compound 11 (560). Nonetheless, compounds 7 and 11 did not select for novel DRMs and both displayed genotypic and phenotypic resistance profiles similar to RPV.

Contrary to RPV and compounds 7 and 11, compound 13 selected HIV-1 clones with two mutations in RT: G112D and M230I. Our data indicate that G112D enhances both resistance to

NNRTIs and replication capacity of virus also containing the M230I mutation. The resistance phenotype of G112D/M230I is unique as these mutations together conferred >10-fold resistance to all NNRTIs and increased susceptibility to some NRTIs and a pyrophosphate analog. This phenomenon was also seen with the I132M RT mutation, which was resistant to NVP but hypersusceptible to the NRTIs TFV and lamivudine (563). RT polymerization assays performed by our collaborators confirm the resistance of M230I and G112D/M230I to compound 13 and susceptibility to PFA (data no shown). The selection of these mutations and their resistance phenotype suggests that compound 13 has a different mechanism of action than RPV and the other analogs.

While compound 13 likely binds the NNRTI-binding pocket, the change from a pyrimidine moiety in RPV to a purine moiety (reversed in compound 11) may also exert an effect on dNTP binding and incorporation, which is not see in other NNRTIs. G112D is located near the conserved D110 and YMDD motif necessary for catalysis during reverse transcription (564, 565). Pressure in this region from compound 13 binding to RT may explain the development of G112D. Interestingly, this mutation showed high resistance to FTC but not other NRTIs. FTC resistance is associated with the M184 position, which is part of the YMDD motif (313, 564). M184I/V mutations increase selectivity for the natural dNTP over FTC because branching of isoleucine and valine side chains hinders incorporation of the drug (566, 567). Therefore, it is possible that G112D provides some level of steric hindrance that reduces FTC efficacy or confers a selective advantage of natural dNTPs over FTC. G112D and G112D/M230I HIV-1 were also hypersusceptible to AZT and PFA, which is unique because DRMs associated with these drugs tend to be antagonistic (568-570). However, HIV-1 with mutations Q151I/V has been shown to be hypersusceptible to both AZT and PFA (570). The Q151 residue resides in the

RT motif responsible for binding the viral template strand and dNTPs during reverse transcription, and specifically contacts the viral genomic template (565, 570). Therefore, the similar hypersusceptible phenotypes of Q151I/V HIV-1 and G112D/M230I HIV-1 further suggest that compound 13 affects dNTP processing in a manner unique from other NNRTIs.

To elucidate the function of these mutations in RT and their resistance mechanism, biochemical assays and modeling should be performed. DNA- and RNA-dependent DNA polymerase assays performed by our collaborators indicate a defect in polymerization of RTs with the M230I mutation, whereas polymerization of G112D RT is comparable to WT RT (data not shown). To determine the effects of RT inhibitors on RT with G112D, M230I, or G112D/M230I mutations, polymerization assays should be repeated in the presence of drugs to confirm the resistance or hypersusceptibility phenotypes we report in viral assays. Single step incorporation assays could also be implemented to determine if the RT mutations confer preference for the NRTIs or natural dNTPs, potentially pointing to the mechanisms of NRTI hypersusceptibility or resistance. However, modeling of compound 13 in RT, as has been completed for compounds 7 and 11 (543, 560), should be performed to predict the mechanism of resistance. Modeling could show how the difference in structures between compound 13 and RPV could affect drug-RT contacts. For example, the presence of the purine moiety in compound 13 most likely alters the H-bond and water binding observed with RPV and the K101 and E138 positions, respectively, as is seen with compound 11 (560). M230 is located in the primer grip region of RT and stabilizes primer residues by forming a hydrogen bond between the amino acid and a phosphate of the primer strand (565). Modeling of compound 13 and RT could show if the drug-enzyme interaction disrupts the primer bond or if M230I sterically hinders the binding of compound 13. Modeling can be used to predict drug-RT interactions, the mechanisms

of resistance, and identify alternative DRMs to guide the refinement of more analogs; however, crystal structures of compound 13 bound to WT and mutant RT are needed to truly understand the inhibitory effects of compound 13 and how the DRMs reduce compound 13 efficacy.

Overall, our data show that the RPV analogs 7 and 11 do not select different DRMs than RPV. The resistance phenotypes of known RPV-associated DRMs of these compounds are similar to RPV as well, results that are supported by our collaborators (560). Yet, compound 13 selected a novel combination of DRMs in HIV-1. The selection of two resistance mutations, one inside the NNRTI-binding pocket (M230I) and one outside (G112D), suggests that compound 13 is causing a conformational shift in RT that is unique to this molecule. Nevertheless, G112D/M230I confers resistance to all clinically approved NNRTIs but results in hypersusceptibility to NRTIs and a pyrophosphate analog. Compound 13 is not an ideal candidate for further development as ART or PrEP because of NNRTI cross-resistance. However, biochemical and modeling experiments to better understand the mechanisms of resistance and hypersusceptibility conferred by G112D and M230I are the next step in understanding the unique effect that compound 13 has on RT and how the structure can be best used to design new drugs that do not select cross-resistant DRMs.

6.0 FINAL DISCUSSION AND IMPLICATIONS FOR PUBLIC HEALTH

The global response to the HIV-1 pandemic is one of the most organized and well-funded efforts to control a human disease; however, 30 years after the discovery of the virus, there is no vaccine or cure. Until a vaccine is approved for public use, PrEP with antiretroviral drugs is an effective method to reduce HIV-1 incidences in populations at high-risk of being infected with the virus. Daily oral Truvada is currently the only PrEP regimen available for prescription, but its global availability is limited to less than 12 countries at the time of this writing. Despite significant reductions in HIV-1 infections by Truvada in multiple clinical trials, the biggest barrier to PrEP efficacy is adherence. Alternative PrEP modalities that address adherence include intercourse-dependent oral Truvada or microbicide gels, IVRs, and long-acting injectables. Intercourse-dependent PrEP and IVRs have shown success in clinical trials, but these options are user-driven and adherence cannot be guaranteed. In contrast, long-acting injectable PrEP would guarantee adherence but the ability of such a strategy to prevent HIV-1 transmission has not been tested in clinical trials.

RPV LA is the long-acting injectable formulation of the NNRTI RPV and is safe in humans and a single dose can provide concentrations above a theoretical protective concentration for at least one month post-dosing. However, the current paradigm of using antiretroviral drugs approved for both ART and PrEP has two issues that should be addressed for every PrEP modality. The first issue is selection of drug resistance in individuals who receive PrEP when

already HIV-1^{+} or in those who experience a breakthrough infection while on PrEP. The second issue is the ability of PrEP to prevent transmission of drug-resistant HIV-1. Thus, the overall concern is an increase in newly infected individuals with drug-resistant HIV-1. Importantly, certain mutations lead to cross-resistance to some or all drugs of the same class (16), thus ART options could be reduced in newly infected individuals should they acquire a virus with DRMs. There is no ethical way to directly study these scenarios in humans; nevertheless, we used two distinct animal models to address two questions in regard to RPV LA as PrEP and its role in resistance selection. One, would RPV LA monotherapy select for DRMs in macaques infected with RT-SHIV (Chapter 3); and two, can RPV LA prevent mucosal transmission of drug-resistant HIV-1 in humanized mice (Chapter 4)? The success of PrEP in clinical trials likely guarantees its eventual global use, thus questions of drug resistance and PrEP efficacy are valuable to study in pre-clinical models prior to large-scale human trials and widespread clinical approval. The public health significance of this dissertation work is the development of pre-clinical animal models to assess the potential consequences of RPV LA use in scenarios that have occurred in clinical trials with Truvada (318-320) and how drug resistance affects PrEP efficacy. Testing these questions in animal models provides a better understanding of RPV LA PrEP, and the results can be used to inform future study designs with the drug.

Due to cross-resistance between NNRTIs (265) and growing global coverage of ART, the utility of NNRTIs is diminishing because of increases in NNRTI-resistance prevalence and transmission (285, 352, 353). Thus, new antiretroviral drugs to expand options for ART and PrEP are needed. These novel drugs should be active against drug-resistant HIV-1, have novel mechanisms of actions, and should not select cross-resistant mutations (or preferably no resistance at all). We were provided with analogs of RPV that are active against known drug-

resistant HIV-1 molecular clones (543). Our objective was to determine the resistance genotype and phenotype of HIV-1 selected in the presence of the analogs as a way to understand the mechanism of action of the compounds (Chapter 5). Mutations that develop under drug selection and confer resistance in a virus can indicate where the inhibitors bind to suppress the HIV-1 replication cycle. The public health significance of this study is a better understanding of novel compounds that can be used to focus the design of new antiretroviral compounds for control of the HIV-1 pandemic.

6.1 RPV LA MONOTHERAPY DOES NOT READILY SELECT FOR RESISTANCE MUTATIONS IN RT-SHIV-INFECTED MACAQUES

The goal of this aim was to address the concern that RPV LA could select for drug resistance should individuals receive the drug after becoming infected with HIV-1. In this situation RPV becomes monotherapy, which is a strategy no longer in practice due to rapid selection of drug-resistant HIV-1 (268-270, 272). In successful Truvada clinical trials, 17 individuals were found retrospectively to be infected with HIV-1 before receiving PrEP, and five of them (29.4%) developed DRMs (318-320). Although providing PrEP to HIV-1^{+} participants was rare, these findings confirm that PrEP can select drug resistance in persons with undetected HIV-1 infection, hence the need to test DRM selection in a monotherapy setting for RPV LA and any other PrEP strategy.

Contrary to earlier studies in macaques infected with RT-SHIV and treated with EFV or NVP monotherapy (389, 465-467), RPV LA monotherapy did not select DRMs at a high or consistent frequency. Two different RPV-associated resistance mutations were detected at a

lower than expected frequency in RT-SHIV isolated from plasma samples of two macaques. No DRMs were observed in virus isolated from ileum or axillary LN tissues of the same macaques, in which drug concentrations were >4-fold higher than in plasma. However, one of 66 participants in a Phase I clinical trial of RPV LA became infected with HIV-1 after receiving a single 300 mg dose of RPV LA (338). This person developed detectable levels of HIV-1 with a RPV-resistant mutation in plasma when drug concentrations were around the $PAEC_{90}$ for RPV (351). In humans, low concentrations of RPV can be detected in plasma and tissues for up to 160 days after a single dose of RPV LA (338, 348). During viral rebound, drug concentrations in our macaques were similar to concentrations reported in the infected person, but resistance selection did not occur in our animals. While our dosing strategy reflected drug concentrations reported in humans, the long tail that hovers around the $PAEC_{90}$ value may be the ideal situation for resistance selection in humans but not in macaques. Our data support that selection of drug resistance by RPV LA monotherapy may be rare in pigtailed macaques infected with RT-SHIV. While our results are based on a population of two animals, single-genome sequencing of HIV-1 RNA isolated from plasma of BLT mice treated with RPV LA PrEP showed similar low frequencies of DRMs (Chapter 4), furthering supporting our macaque results. Nonetheless, the experiment could be repeated with a larger cohort of macaques to show consistency with the RPV LA/RT-SHIV model and eliminate idiosyncrasies of the two animals that may have affected the results.

Our data support that RPV LA monotherapy does not easily select for resistance mutations in RT-SHIV-infected macaques or HIV-1-infected humanized mice. Similar to RT-SHIV, we had difficulty selecting for RPV resistance in HIV-1-infected cells *in vitro* (Chapter 5). RPV concentrations >100-fold the *in vitro* EC_{50} were needed to select for mutant viruses with

single low-level DRMs. Overall, our data support that low concentrations of RPV do not readily select DRMs in RT-SHIV (or HIV-1); thus, RPV LA may not significantly increase the prevalence of DRMs if approved for widespread prophylactic use. However, the contrasting outcomes between our two macaques on RPV LA monotherapy and one individual on RPV LA with a breakthrough infection should be further studied to better understand the consequences of RPV LA use.

6.2 RPV LA CAN INHIBIT DRUG-RESISTANT HIV-1 TRANSMISSION IN BLT MICE BUT NOT AT BIOLOGICALLY RELEVANT DRUG CONCENTRATIONS

The goal of this aim was to determine the efficacy of RPV LA to inhibit mucosal transmission of WT and drug-resistant HIV-1. RPV LA has been shown to be safe and tolerable in multiple clinical trials and provide drug concentrations above a theoretical protective $PAEC_{90}$ value for at least a month post-dosing (338, 340, 348). To date, only one study has tested the ability of RPV LA to inhibit mucosal transmission of HIV-1 infection. Kovarova *et al*. showed that a single dose of RPV LA protected 13/16 (81%) mice from vaginal infection after challenge with WT transmitted/founder isolates of HIV-1 (501). However, the RPV LA dose used in this study resulted in sustained plasma RPV concentrations for 28 days post-injection approximately 6-fold higher than peak concentrations reported in humans (six days post-injection) who received a single 1,200 mg dose of RPV LA (338) and tissue RPV concentrations were not measured. In contrast, we performed PK analysis of RPV LA in plasma and mucosal tissues and found that a 150 mg/kg IM dose of RPV LA in BLT mice recapitulated RPV concentrations reported in humans, thus providing a more biologically relevant model for efficacy studies.

We found that biologically relevant concentrations of RPV did not significantly inhibit vaginal transmission of WT or low-level resistant HIV-1. However, we then challenged mice when plasma and vaginal tissue RPV concentrations were approximately 8-fold higher than concentrations reported in humans, similar to Kovarova *et al*. (501). With this dosing strategy, we observed total protection from vaginal infection by low-level resistant HIV-1 (Y181C), demonstrating that RPV LA can inhibit vaginal transmission of virus with a DRM. This is an encouraging result, as most known RPV-associated DRMs confer low-level resistance to the drug, similar to Y181C (265). However, we did not observe statistically significant protection from WT or high-resistant HIV-1. We believe the lack of protection to WT HIV-1 is due to the high replication rate of the lab-adapted challenge strain. It is possible that the use of transmitted/founder HIV-1 isolates and a different titer method by Kovarova *et al*. (501) resulted in a significant difference between the challenge inoculums used between our studies. It is possible the modest replication defect conferred by Y181C in our challenge stock created a virus with similar replication capacity to the WT viruses used by Kovarova *et al*. (501), hence the similar protective results. *In vitro* infectivity and replication assays could directly assess the dissimilarities between our lab-adapted and the transmitted/founder challenge stocks used in the experiments. While our study was designed to analyze specific mutations within RT, we plan to use clinical WT and drug-resistant HIV-1 isolates in future experiments to determine what concentrations of RPV LA should be achieved to inhibit mucosal transmission. The use of clinical isolates would allow us to elucidate the effect of RPV LA concentrations on transmission of biologically relevant viruses; however, the use of clinical isolates introduces the issue of mutations outside of RT and heterogeneous populations that could influence results by affecting infectivity and replication capacity. Therefore, cloning the RT region, and perhaps the Env

region, from clinical viruses into a molecular clone of HIV-1 with consistent replication is being considered to reduce such confounding factors. Regardless, *in vitro* infectivity and replication will be measured to normalize challenge doses.

Although RPV LA did not provide the level of protection against WT HIV-1 that we had hypothesized, detection of plasma viremia was delayed in two WT-infected mice challenged at high RPV concentrations. This indicates that RPV LA had a suppressive but not protective effect on early virus replication. We could utilize animals with this "smoldering" infection to identify sanctuary sites and track initial HIV-1 spread through visualization techniques such as bioluminescence or PET imaging, similar to what has been performed with SIV in mice (571) and macaques (572). Although we have PK data for mucosal tissues, there could be an uneven temporal distribution of active RPV that would allow sanctuary sites to form and be maintained until systemic drug concentrations decline to allow viral circulation. Imaging studies combined with PK analyses could elucidate the specific location(s) of low-level virus replication and determine what drug concentrations are needed to prevent mucosal transmission. For example, if we identify a specific tissue as a sanctuary site, we could then perform a dose response study with RPV LA until we find the drug concentration that prevents such sites from forming. It is also possible that virus is shielded from RPV by immune cell interactions, such as capture by dendritic cells (573), and *ex vivo* immunofluorescence assays could identify the cell populations and tissue locations responsible for such a phenomenon. This information could guide future RPV LA or PrEP designs to account for dosing, improved systemic drug dissemination, and specific cell/tissue drug targeting. Work has begun on the development of infectious and replication competent reporter viruses that produce nano-luciferase or infrared fluorescent protein for *in vivo* imaging of active virus replication sites.

Despite breakthrough infection in the majority of mice challenged at day 7 post-RPV LA dosing, no DRMs were detected in plasma virus by population sequencing. Subsequent single-genome sequencing of a larger group of infected mice revealed a low prevalence of NRTI- and NNRTI-associated DRMs in both untreated and RPV LA-treated animals. The low frequency of DRMs bolsters our results from Chapter 3 that RPV LA does not readily select for drug resistance but is contradictory to the infection of a participant in the SSAT 040 trial (351). Although we did not perform PK analysis beyond week 1 post-RPV LA injection, it is likely that RPV in our mice would still be detectable at concentrations similar to the infected human through the end of our study.

It is also possible that physiological differences between humans and mice, such as metabolism or protein binding, account for the discrepancy in protective efficacy and resistance selection. To accurately test this, we would need to perform an equilibrium dialysis assay to determine the amount of free, active RPV in mice and humans in relevant tissue compartments; however, this would be costly and technically challenging. Alternatively we could perform drug susceptibility assays in the presence mouse plasma to calculate a $PAEC_{90}$ to better predict protective RPV concentrations in mice. Based on our PK data, we could adjust dosing strategies to define protective levels of RPV or determine suboptimal concentrations that would be more likely to select for resistance. Thus, longitudinal studies with different doses of RPV LA or challenges at different time points may better reveal the effect of RPV on resistance selection and further refine the BLT model for RPV LA and future PrEP studies.

Combined with results from human tissue explant models (348, 349), our preliminary data comparing vaginal or rectal transmission of WT HIV-1 in RPV LA-treated mice indicate that the drug at current proposed dosing regimens may be more effective at preventing rectal

rather than vaginal HIV-1 transmission. However, we need more animals to provide statistical power to the analysis. Even if RPV LA is more appropriate for prevention of rectal infection, we still want to know what concentrations of RPV are protective. To determine these protective concentrations *in vivo*, we could take plasma samples on the day of challenge for pharmacodynamic analysis to establish a protective correlation between plasma and mucosal tissue drug concentrations. This could determine a protective threshold for RPV, as was done for CAB LA in macaques (484). However, it may be worth utilizing the RT-SHIV macaque model for such a study if we first addressed the metabolism of RPV as discussed in the previous section. Using macaques would allow for larger plasma volumes for more sensitive virus detection and mucosal tissue biopsies to correlate actual RPV concentrations at the site of infection. Knowing what concentrations of RPV are necessary for effective HIV-1 inhibition at mucosal sites would provide a better understanding of RPV tissue deposition, guide dosing strategies, and could influence selection of the target populations in future RPV LA trials.

Overall, our data indicate that RPV LA can be effective at preventing low-level drug-resistant (2-fold) HIV-1 infection in BLT mice, but not at concentrations observed in human women and not against HIV-1 with higher RPV resistance (27-fold). It will be important to determine an accurate *in vivo* protective concentration of RPV in plasma and mucosal tissues, rather than relying on a theoretical $PAEC_{90}$ based on *in vitro* data. With a more accurate *in vivo* protective value and clinical HIV-1 isolates, we could design challenge and resistance selection experiments that closely recapitulate human exposure events and provide results that could shape RPV LA dosing recommendations and clinical trial design.

6.3 A RPV ANALOG SELECTS FOR A NOVEL COMBINATION OF MUTATIONS IN HIV-1 REVERSE TRANSCRIPTASE

The goal of this work was to determine the resistance profile of three analogs of RPV. By knowing what DRMs these compounds select, we may be able to predict where they bind and how they inhibit HIV-1 replication. The flexible chemical structure of RPV allows it to adjust to mutations in RT (288) and is credited with the low-level resistance conferred by many DRMs (265). Therefore, the structure of RPV was altered to create analogs that potentially select novel DRMs in HIV-1 while maintaining the safety and potency associated with RPV (543). Utilizing *in vitro* assays, we determined the resistance genotype and phenotype of HIV-1 exposed to the RPV analogs and found that one selected a novel combination of RT mutations that conferred resistance to NNRTIs, but was hypersusceptible to NRTIs.

While two of the analogs selected RPV-associated DRMs with similar resistance genotypes and phenotypes as RPV, compound 13 selected for the combination of G112D and M230I mutations in RT. This is unique as currently approved NNRTIs typically select single amino acid changes. The presence of both RT mutations in HIV-1 enhanced replication capacity and NNRTI-resistance compared to virus with either mutation alone. Interestingly, the G112D mutation conferred resistance to the NRTI FTC, no resistance to NNRTIs, and hypersusceptibility to the NRTI AZT and the pyrophosphate analog PFA. Selection of G112D is unique since all DRMs associated with FDA-approved NNRTIs exist near or within the NNRTI-binding pocket, but G112D is near the D110 catalytic domain of the polymerase active site. This indicates that the structure of compound 13 may be binding RT in a novel manner and exerting pressure on RT outside of the canonical NNRTI-binding region. While the M230I mutation is considered part of the NNRTI-binding pocket and conferred cross-resistance to clinically

relevant NNRTIs, this mutation also conferred hypersusceptibility to the NRTIs we tested, which may explain why M230I is not prevalent in sequences cataloged in the HIV Drug Resistance Database (266). To further elucidate the inhibitory mechanism of compound 13, we could test compound 13 against a larger panel of RT inhibitor-resistant HIV-1 clones to predict amino acid positions in which the compound may interact with the viral enzyme.

Because G112D and M230I exist in different motifs of RT, it would be fascinating to know how they alter RT structure to preserve enzyme functionality in the presence of NNRTIs. RT polymerization assays have confirmed a processing defect in RT with the M230I mutation, while G112D alone does little to effect enzyme function. Polymerization assays in the presence of RT inhibitors are planned to confirm our resistance phenotype results. Because compound 13 affects NRTI susceptibility, dNTP incorporation assays could also be performed to determine the effect of the mutations on selectivity and incorporation. This would provide evidence to the mechanism of resistance or hypersusceptibility to NRTIs and PFA. Since the chemical structures of compound 13 and RPV are similar, we hypothesize that compound 13 targets the NNRTI-binding pocket, and *in silico* modeling of compound 13, using a RPV-RT crystal structure as a template, could predict potential sites of interaction (and DRMs) between RT and compound 13. Drug-RT interactions could be tested phenotypically as mentioned in the previous paragraph. This assumes that compound 13 behaves in a similar manner as RPV, which may not be the case given the selection of G112D. To truly understand how the compound binds, crystal structures of compound 13 bound to a WT and G112D/M230I mutant RT should be solved. Crystallographic structures would reveal how compound 13 and RT truly interact, complement results of drug susceptibility analyses, and refine *in silico* modeling for future analogs.

Although compound 13 selected for a novel combination of resistance mutations in HIV-1, suggesting a higher genetic barrier to resistance than RPV and the other analogs, the pair of mutations conferred cross-resistance to all clinically prescribed NNRTIs. This cross-resistance makes compound 13 unsuitable to move forward for clinical development. However, the selection of two mutations, one outside of the NNRTI-binding pocket, is unique and suggests that compound 13 is binding in a manner distinct from other NNRTIs, particularly RPV. Thus, compound 13 could serve as the backbone for development of a novel NNRTI or a new class of RT inhibitors that could eventually become approved antiretroviral drugs for ART or PrEP.

6.4 CONCLUSION

The unifying theme of this dissertation is the study of the effects of HIV-1 resistance on the FDA-approved drug RPV in regard to its use as a long-acting PrEP agent or as the backbone for novel antiretroviral compounds. We used animal models to address concerns of resistance selection as a result of RPV LA use and the ability of the formulation to prevent transmission of drug-resistant HIV-1. Our data indicate that RPV LA when administered as PrEP or monotherapy, an unintended consequence of its prophylactic use, does not readily select a viral population with high levels or consistent DRMs. This is an encouraging finding as selection for drug resistance should be negligent in compounds proposed for PrEP use. Also, our humanized mouse model shows that RPV LA can prevent vaginal infection of HIV-1 with a low-level RPV resistance mutation, suggesting the prophylactic formulation should be effective in areas with circulating drug-resistant HIV-1, particularly Y181C the second most prevalent transmitted NNRTI-resistant mutation. However, our data also indicate that RPV LA may not prevent

infection with viruses containing transmitted DRMs that confer higher levels of resistance (*e.g.* Y181V), thus the prevalence of certain DRMs in an area should be considered before administering RPV LA. More importantly, our results show that biologically relevant concentrations of RPV in humans do not translate to protection in humanized mice and protective concentrations between mucosal compartments may be significantly different. While drug metabolism differences between humans and mice may account for this discrepancy, we suggest tempering expectations that the current RPV LA dosing strategy in humans should be effective at significantly protecting at-risk populations from HIV-1 infection. Instead, pharmacodynamic studies should be performed to better define an *in vivo* protective concentration of RPV prior to efficacy trials.

Finally, we determined the resistance profile of three RPV analogs to find where they interact with RT and if they could be of use as future ART or PrEP drugs. One of the compounds selected a novel combination of mutations not associated with other RT inhibitors; however, the two mutations together conferred cross-resistance to FDA-approved NNRTIs suggesting the compound targets the NNRTI-binding pocket in a way unique from the approved drugs. This suggests the structure of the compound could be further modified to create a new NNRTI or even a novel class of RT inhibitors with a distinct resistance profile. While more research should be performed to better understand RPV, the data presented in this dissertation demonstrates the utility of RPV for PrEP and as a step toward future anti-HIV-1 drugs.

APPENDIX: ABBREVIATIONS PRESENTED WITHIN THE WORK

AIDS – acquired immunodeficiency syndrome
AAALAC – American Association of Accreditation of Laboratory Animal Care
APOBEC3 – apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3
ART – antiretroviral therapy
BLT – bone marrow, liver, thymus
CAB – cabotegravir
CAB LA – long-acting cabotegravir
CCR5 – C-C chemokine receptor type 5
CD3 – cluster of differentiation 3
CD4 – cluster of differentiation 4
CD8 – cluster of differentiation 8
CD34 – cluster of differentiation 34
CD45 – cluster of differentiation 45
cDMEM – complete Dulbecco's modified Eagle's medium
cDNA – complementary deoxyribonucleic acid
CPE – cytopathic effect
CRF – circulating recombinant form
CXCR4 – C-X-C chemokine receptor type 4
DAP – dapivirine
DMEM – Dulbecco's modified Eagle's medium
DNA – deoxyribonucleic acid
dNTP – deoxynucleoside triphosphate
DMPA – depot medroxyprogesterone
DRM – drug resistant mutation
EC_{50} – effective concentration to prevent 50% infection
EC_{90} – effective concentration to prevent 90% infection
EFV – efavirenz
Env – HIV-1 envelope protein
ETR – etravirine
FBS – fetal bovine serum
FDA – Food and Drug Administration
FTC – emtricitabine
GALT – gut-associated lymphoid tissue
HIV-1 – human immunodeficiency virus type 1
HSV-2 – herpes simplex virus type 2
IACUC – Institutional Animal Care and Use Committee

IL-2 – interleukin-2
IM – intramuscular
IU – infectious units
IV – intravenous
IVR – intravaginal ring
LC-MS/MS – liquid chromatograph-tandem mass spectrometry
LN – lymph node
MOI – multiplicity of infection
mRNA – messenger RNA
MSM – men who have sex with men
MVC – maraviroc
NIH – National Institute of Health
NK – natural killer
NNRTI – non-nucleoside reverse transcriptase inhibitor
NRTI –nucleoside reverse transcriptase inhibitor
NSG – NOD-*Prkdc*^{scid} *IL2rg*^{Tm1Wjl}
NVP – nevirapine
$PAEC_{90}$ – protein-adjusted effective concentration to prevent 90% of infections
PBMC – peripheral blood mononuclear cells
PBS – phosphate buffered saline
PFA – foscarnet
PIC – pre-integration complex
PK – pharmacokinetic
PrEP – pre-exposure prophylaxis
QC – quality control
qRT-PCR – quantitative reverse transcriptase PCR
RAL – raltegravir
RLU – relative light unit
RNA – ribonucleic acid
RPV – rilpivirine
RPV LA – long-acting rilpivirine
RT – reverse transcriptase
Pol II – human RNA polymerase II
scid – Severe combined immunodeficiency
SHIV – simian-human immunodeficiency virus
siRNA – small interfering ribonucleic acid
SIV – simian immunodeficiency virus
T-20 – enfuvirtide
TAR – transactivation-response
TFV – tenofovir
US – United States
vDNA – viral deoxyribonucleic acid
vRNA – viral ribonucleic acid
WHO – World Health Organization
WT – wild-type

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