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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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Preexposure prophylaxis (PrEP) using antiretroviral drugs is effective in reducing the risk of human immunodeficiency virus type 1 (HIV-1) infection, but adherence to the PrEP regimen is needed. To improve adherence, a long-acting injectable formulation of the nonnucleoside reverse transcriptase (RT) inhibitor rilpivirine (RPV LA) 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 simian immunodeficiency virus containing HIV-1 RT (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 only at one time point, and no resistance mutations were detected in viral RNA isolated from tissues. Wild-type 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 injection of RPV LA.

Despite increasing use of antiretroviral therapy (ART), which suppresses viral replication and reduces the risk of human immunodeficiency virus type 1 (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, preexposure prophylaxis (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 tenofovir (TDF) with or without emtricitabine (FTC) (2–5). These successes led to FDA approval of daily oral TDF-FTC PrEP for high-risk populations.

One prominent issue with PrEP is dependence of efficacy on adherence. Data from clinical trials has shown that patients with detectable plasma drug levels indicative of adherence have reduced risk of HIV-1 infection, while poor PrEP adherence confers little protection (2, 4, 6–8). Although recent work suggests that sexual event-driven TDF-FTC administration can be effective in men (9–11), sporadic PrEP adherence can lower PrEP efficacy and promote selection of drug-resistant variants. To reduce pill burden, long-acting injectable nanoparticle formulations of the nonnucleoside reverse transcriptase inhibitor (NNRTI) rilpivirine (RPV) and an experimental integrase inhibitor, cabotegravir, have been developed (12, 13). 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 (14–17).

Rilpivirine 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 (18, 19). Clinical trials have shown RPV-TDF-FTC to be noninferior to and more tolerable than efavirenz-based ART (18). During these trials, patients failing RPV-based ART also tended to select unique NNRTI-associated resistance mutations in reverse transcriptase (RT) compared with those on an efavirenz-based regimen (20). Long-acting rilpivirine (RPV LA) is an injectable nanoparticle formulation and has been shown to be safe and tolerable, with detectable drug concentrations maintained in plasma and tissues weeks after a single injection (21–24). Multiple clinical trials are under way to test safety and acceptability of RPV LA as PrEP in HIV-1-uninfected men and women (13).

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 drug-resistant mutations (DRMs), promoting spread of drug resistance and limiting future ART options through cross-resistance (25). Resistance analyses of breakthrough infections in TDF-FTC

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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 (26–28).

Although the development of DRMs during TDF-FTC PrEP clinical trials was rare (26–28), there are 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 simian immunodeficiency virus (SIV) that contains the HIV-1 reverse transcriptase coding region (29, 30). 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 (31, 32). RT-SHIV macaque models have been used to study HIV-1 ART, drug resistance, PrEP, and persistence (30, 32–48). 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*.

MATERIALS AND METHODS

Cell culture and antiretroviral inhibitors. 293T and TZM-bl (49) 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). GHOST-R3/X4/R5 cells (50) were maintained in the same medium as described above with the addition of 100 μg/ml of Geneticin (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 P-S-G. All cell lines were incubated at 37°C with 5% CO_2.

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

Virus production and titer determination. Plasmids encoding HIV-1_{LAI} with silent restriction sites within RT (51) and RT-$SHIV_{mne}$ (30) 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). HIV-1 titers were determined on GHOST cells with modifications from previous work (50), using an LSRII flow cytometer (BD Biosciences). Titers of RT-SHIV stocks were determined using TZM-bl indicator cells and β-galactosidase staining as previously described (29, 52).

Animals. Two juvenile pigtailed macaques (*Macaca nemestrina*) were housed at the University of Pittsburgh in accordance with American Association of Accreditation of Laboratory Animal Care standards. All procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee. 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 intravenously with 1×10^5 infectious units (IU) of RT-$SHIV_{mne}$. At 6 and 8 weeks postinfection, animals were treated with 200 mg/kg of RPV LA by intramuscular (i.m.) injection near the scapula. This dosing regimen was determined by prior pharmacokinetic 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 h 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 postinfection, and multiple tissues, including axillary lymph nodes (LN) and ileum, were immediately flash frozen and stored in liquid nitrogen.

T cell counts and viral RNA isolation. Plasma was separated from EDTA-treated whole blood by centrifugation at 400 × *g* for 10 min and stored at −80°C until processed. Viral RNA (vRNA) was isolated from plasma as previously described (53). Briefly, plasma was mixed with Tris-buffered saline (Sigma) and pelleted by centrifugation at 21,000 × *g* for 1 h. Virus was then suspended in 3 M guanidinium chloride, 50 mM Tris-HCl (pH 7.6), 1 mM $CaCl_2$, and 1 mg/ml of proteinase K and incubated at 42°C for 1 h, 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 8.0). Tissue vRNA isolation was performed as previously described (54).

Peripheral blood mononuclear cells (PBMCs) were isolated from samples using lymphocyte separation medium (Corning) and treated with ACK lysis buffer (Life Technologies) to remove red blood cells. PBMCs were stained with NHP T lymphocyte cocktail (BD Biosciences) with antibodies against 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.

RPV plasma and tissue levels. Rilpivirine 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 nanograms per milliliter. 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%.

Viral RNA quantitation. Viral RNA was measured by generation of cDNA from vRNA isolated from plasma or tissues by the SuperScript III first-strand synthesis kit (Life Technologies) with the SIVgag-R primer (5′-CACTAGGTGTCTCTGCACTATCTGTTTTG-3′). Quantitative PCR was performed on cDNA using SsoFast probes SuperMix (Bio-Rad), SIVgag-F (5′-GTCTGCGTCATCTGGTGCATTC-3′) and SIVgag-R primers, and the SIVgag-probe (5′-FAM-CTTCCTCAGTGTGTTTCACTTTCTCTTCT-GCG-3′ 6-carboxytetramethylrhodamine [TAMRA]). CCR5 primers and probe were previously described (54). 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.

Viral RNA sequencing. The RT coding region was amplified from viral cDNA 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′-GCAAAAGGATTAAAGGGACA

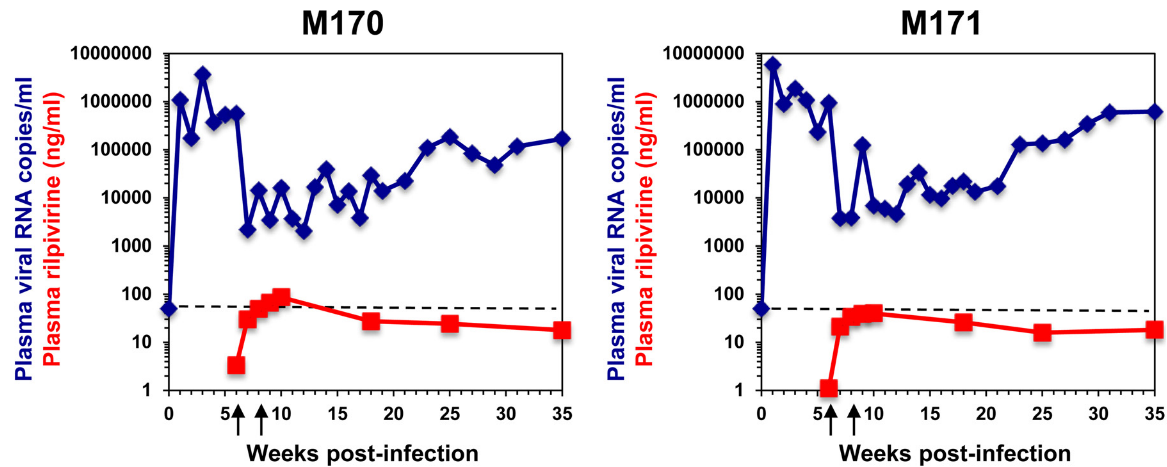

FIG 1 Plasma RT-SHIV levels in two macaques treated with RPV LA monotherapy. Animals were infected at week 0 and received two i.m. doses of RPV LA at 6 and 8 weeks postinfection (designated by arrows under the *x* axis). Plasma viremia (blue lines) and RPV concentrations (red lines) were measured. The dashed lines indicate the limit of detection of the qRT-PCR assay (50 RNA copies/ml).

A-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 DNASTAR (Lasergene).

RT-SHIV *in vitro* resistance selection. CEMx174 cells were infected with wild-type (WT) RT-SHIV at a multiplicity of infection (MOI) of 0.05 in medium and incubated at 37°C for 2 h. 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 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.

Drug susceptibility assay. Drug susceptibility assays were performed as previously described, with minor modifications (29). TZM-bl cells were seeded at 5×10^3 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 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 h. Luciferase activity was measured using Britelite Plus reagent (PerkinElmer) on a Luminoskan Ascent microplate luminometer (Thermo Scientific). Relative luciferase 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 effective concentration to inhibit 50% of virus replication (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.

RESULTS

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 1×10^6 and 1×10^7 copies of vRNA/ml, which declined to a set point of 5×10^5 to 9×10^5 vRNA copies/ml (Fig. 1). Based on pharmacokinetic results (data not shown), animals were treated with two 200-mg/kg i.m. doses of RPV LA at weeks 6 and 8 postinfection. Posttreatment, there was an immediate ~2-log decline in plasma viremia, which was maintained at approximately 1.35×10^4 vRNA copies/ml until 21 weeks postinfection. Viral loads slowly rebounded thereafter to pretherapy set point values (Fig. 1). There was a transient decrease in the percentage of $CD4^+$ T cells in both animals after infection, followed by a steady posttreatment 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.

Although a standard protective level of RPV has not been defined, the *in vitro* EC_{50} of RPV is 0.1 to 0.7 nM (55, 56), and our dosing strategy was designed to achieve plasma RPV concentrations equivalent to that reported for humans: 50 ng/ml (136 nM) (22, 56). 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 postinfection in animals M170 and M171, respectively (Fig. 1). 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 postinfection) (Table 1). As tissue homogenate concen-

TABLE 1 RPV concentrations in plasma and tissues of animals at 35 weeks postinfection

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

TABLE 2 Mutations identified in RT of plasma RT-SHIV RNA

Animal	Mutations in RT-SHIV RT[a] (no. of clones)				
	Wk 6[b]	Wk 9	Wk 13	Wk 21	Wk 25
M170	WT (14) Non-DRM (31)	WT (25) Non-DRM (19)[c] R125G, E138G (1)	WT (19) Non-DRM (25) S68G, <u>E138G</u> (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) <u>E138Q</u>, P157-, L213F, V244C (1)

[a] Results represent a mixture of full-length and partial (amino acids 1 to 250) RT sequences. WT, wild type; non-DRM, mutations not associated with drug resistance; -, codon deletion; underline, known RPV-associated resistance mutations.
[b] Prior to RPV-LA administration (6 and 8 weeks postinfection).
[c] One sequence at this time point for this animal contained A33V.

trations are averaged across a heterogeneous matrix, these data do not differentiate between extracellular or intracellular drug exposure or intracellular drug exposure between cell types. Therefore, the averaged concentration could significantly overrepresent 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.

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 postinfection. 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 2). 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, 45 clones were sequenced at 6, 9, 13, and 25 weeks postinfection. 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 3). 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 (55, 57).

TABLE 3 RPV susceptibilities 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	1.0
	E138G	0.94 ± 0.03	1.4
	E138Q	2.85 ± 0.08	4.3
	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	1.0
	E138G	0.76 ± 0.12	2.2
	E138Q	0.30 ± 0.05	0.8

[a] Values are means and standard deviations from 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.

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 postinfection was clonally sequenced. Axillary LN 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 (58). High levels of RT-SHIV RNA copies were measured by quantitative reverse transcription-PCR (qRT-PCR) in both the axillary LN and ileum taken at necropsy from both animals (Table 4). 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 1).

No known DRMs were detected in 20 clones from either tissue

TABLE 4 RNA copies of RT-SHIV *gag* and macaque *CD4* measured from tissue RNA

Animal	Tissue[a]	No. of copies[b]		*gag*/*CD4* ratio
		gag	*CD4*	
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 postinfection.
[b] Means ± standard deviations ($\times 10^6$), measured by qRT-PCR in duplicate.

TABLE 5 Mutations identified in RT of RT-SHIV RNA isolated from tissues

Animal	Tissue[a]	Mutation(s) 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 postinfection.
[b] Sequences represent amino acids 1 to 250 of RT. WT, wild type; ., stop codon.

from both animals (Table 5). 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 2). 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 (55). 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* (K. Melody and Z. Ambrose, unpublished data).

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 HIV-1 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 3).

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* (55), 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 (55), 25% human serum reduced RPV

TABLE 6 Effects 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
	25% macaque	3.17 ± 1.37	8

[a] Means ± standard deviations from triplicates from one experiment.
[b] Compared to assay performed with 10% FBS.

inhibition of HIV-1 *in vitro* in our study 12-fold (Table 6). Similarly, addition of macaque serum increased the EC_{50} of RPV against RT-SHIV compared to the values with medium with FBS (Table 6). 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 nevirapine (NVP) or efavirenz (EFV), respectively (29).

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 (2–5, 7). 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 (2, 4, 6, 8, 59, 60). To improve adherence, long-acting injectable nanoparticle formulations of RPV have been developed and are currently being evaluated in clinical trials (13). 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 (61).

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 (30). While RPV LA dosing of and metabolism in macaques were different than those 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 (22, 24). RPV LA treatment displayed a therapeutic effect with approximate 2-log decreases in plasma

viremia 1 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 lymph nodes than in plasma (21). 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 completely 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 4 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 (30, 40, 62). Another study using a different strain of RT-SHIV in rhesus macaques also showed that K103N and other DRMs arise during EFV monotherapy (34). The RT mutation K103N confers approximately 20- to 35-fold resistance to HIV-1 *in vitro* and arises in HIV-infected individuals on EFV-based ART (55, 63).

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 (55). K103N was not detected in patients failing RPV-containing ART in two clinical trials (20). In fact, with the exception of K101P, Y181I, andY181V, 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 (55). 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 (20, 55, 64, 65), which is less likely to occur prior to therapy or to be transmitted to newly infected individuals (66). 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 intravaginal rings (IVR) 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 (39). One IVR-treated animal had a single plasma clone containing Y181I, while all other clones were WT. Similar results were found in LN 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 pharmacokinetics of the drug in HIV-negative individuals (67). 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 (20, 68). 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 (69). This interaction was lost with the substitution of the low-level RPV-resistant mutations 138A/G/K/R/Q or 101E, suggesting that disruption of the K101-E138 salt bridge causes low-level RPV resistance (70). 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 (71) or G-to-A hypermutation (72). 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 levels. 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.

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