Supporting Information

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SI Materials and Methods

Study Population. Between June 2009 and July 2012, a total of 12 subjects were enrolled into a protocol in which antiviral therapy (ART) was initiated after obtaining peripheral blood (PB), inguinal lymph node (LN), and ileum and rectal biopsies via colonoscopy. Subjects were required to be antiretroviral naïve or to have been off any antiretroviral agent for at least 1 y. All subjects gave written informed consent, and the Institutional Review Board of the University of Minnesota approved the study.

Study Procedures. At screening, PB was obtained to determine CD4 T-cell count and plasma HIV RNA, and plasma was sent for genotype and phenotype assay. Just before initiation of ART, all patients had an excisional inguinal LN biopsy and a colonoscopy to obtain ∼14 snip biopsies of the terminal ileum and 14 snip biopsies of the rectum. PB was also obtained. The patient was then started on a Department of Health and Human Services-approved regimen for therapy of HIV infection. The subject and his or her primary HIV provider determined the specific regimen initiated. PB was obtained weekly for 2 wk, again at week 4, and then monthly thereafter to monitor CD4 T-cell count and plasma HIV RNA and to measure PB drug concentrations. Inguinal LN and terminal ileum and rectum samples were obtained again at months 1, 3, and 6 (M1, M3, and M6) after starting ART. At M3, subjects were kept in the research unit for an intensive PK study where blood was obtained at 0, 1, 2, 3, 4, 6, 8, 12, and 24 h after an observed dose of the ART regimen to determine plasma pharmacokinetic characteristics. Subjects were followed in the study for a total of 6 mo after ART initiation.

Clinical and Study Laboratory Procedures for Initial Tissue Management. Initially, HIV-1 viral load testing was performed by using the Siemens assay platform with a limit of detection of 75 copies per milliliter. This platform was used for subjects 1,669; 1,679; and 1,680 through M5, M2, and week 2, respectively; all other samples were analyzed by using the Roche platform that has a limit of detection of 48 copies per milliliter CD4 count, complete blood count, and chemistry panel testing were performed at the University of Minnesota Fairview Clinical Laboratory. Siemens TRUGENE HIV-1 genotype/phenotype assay was used to detect ART resistance.

PB was collected in cell preparation tubes (CPTs) and acid citrate dextrose tubes for PBMC isolation. CPTs were centrifuged within 30 min of collection at 1,800 × *g* for 30 min at room temperature. The layer above the gel was transferred to a 50-mL conical tube and spun at 400 × *g* for 10 min at 4 °C to pellet the cells. Plasma was removed, and cell count was obtained with a hemocytometer. The pellet was washed with PBS before adding 500 μL of cold 70% methanol/30% water to lyse the cells. The samples were stored at −80 °C until shipment to the Antiviral Pharmacology Laboratory (University of Nebraska Medical Center) for measurement of drug concentrations.

At the time of biopsy, LN was dissected into three pieces. Half was transferred to 4% (wt/vol) paraformaldehyde for 4–6 h and then washed in 80% (wt/vol) ethanol and embedded in paraffin. 5-μm sections were used for in situ hybridization. A portion of the remaining LN was flash frozen in liquid nitrogen and subjected to cell disaggregation. Using two scalpel blades, the LN was minced into small pieces then transferred to a 70-μm cell strainer, where it was disaggregated by using the rubber end of a syringe. The cell strainer was washed with medium before spinning cells at 400 × *g* for 10 min. The pellet was washed with PBS, counted with a hemocytometer, and pelleted again. After removing all of the PBS, a portion of the cells were lysed in 500 μL of cold 70% methanol/ 30% water and kept at −80 °C until shipped to the Antiviral Pharmacology Laboratory.

In Situ Hybridization. Five-micrometer-thick sections from the paraffin-embedded tissue were cut through the entire tissue block. We analyzed a total of 20 sections from each block, using every fourth section so that we were measuring virus production in 20-μm intervals. After deparaffinization with xylene and rehydration through graded ethanols, tissue sections were treated with HCl, triethanolamine, digitonin, and 4 μg/mL Proteinase K, as described (1). After acetylation with acetic anhydride and dehydration, they were hybridized at 45 °C overnight with a ^{35}S-labeled riboprobe and 0.5 mM aurintricarboxylic acid in the hybridization mix. After extensive washes and ribonuclease treatment, tissue sections were dehydrated, coated in Ilford K5 emulsion diluted with glycerol and ammonium acetate, exposed at 4 °C for 7–14 d, and developed and fixed per manufacturer's instructions. They were stained with hematoxylin, dehydrated, and mounted with Permount. Photographic images using epifluorescence were taken with a digital camera, and the tif images were analyzed for the area of the sections and the area occupied by silver grains using Photoshop with Fovea Pro. Section weights were estimated from their 5-μm thickness and their area. These methods have been extensively reviewed (2, 3, 4, 5).

Analytical Pharmacology. Lysed cellular matrix from PBMCs, and MNCs obtained from biopsy samples of the LN, ileum, and rectum, was mixed and then centrifuged to facilitate removal of the supernatant for analysis. Tenofovir-diphosphate (TFV-DP) and emtricitabine-triphosphate (FTC-TP) were separated from interferences, metabolites, and unphosphorylated drug with ion exchange solid-phase extraction (SPE). Triphosphates (TPs) were dephosphorylated back to the parent drugs TFV and FTC, then subsequently desalted with reversed-phase SPE, dried and reconstituted. Atazanavir (ATV), darunavir (DRV), ritonavir (RTV), and efavirenz (EFV) were extracted with acidic acetonitrile precipitation and diluted with water to match mobile-phase starting conditions. Final extracts of both methods were quantified with a liquid chromatography–MS system consisting of a Shimadzu Nexera ultra high-performance liquid chromatograph attached to an AB Sciex 5500 QTrap mass spectrometer. Ion-pairs (TFV/FTC/ATV/DRV/RTV, positive; EFV, negative) were monitored in multiple reaction-monitoring mode. Stable-isotope-labeled internal standards (IS) were used for each analyte, and the ratios of analyte to IS were used to determine response. The linear range of quantification for each analyte was 2–400 fmol total mass on column, except for ATV, which was 0.2–40 fmol total mass on column. Quality control sample interbatch coefficients of variance for the TFV-DP and FTC-TP and ATV, DRV, RTV, and EFV methods were 3.84–7.67% and 1.1–7.4%, respectively. Absolute mean relative errors to the theoretical target quality control samples were <5.6% for TFV-DP and FTC-TP and <8.1% for ATV, DRV, RTV, and EFV. Batch acceptance criterion was derived according to the Food and Drug Administration Guidance for Industry on Bioanalytical Method Validation (6). Results from intracellular analysis were expressed in femtomoles per million cells. For any comparison of ATV, DRV, RTV, and EFV concentrations in PBMCs, LN, ileum, and rectum with those concentrations in plasma, a mean cell volume of 282.9 femtoliters (fL) (7) and the appropriate

molecular weight of the drug were used to convert from femtomoles per million cells to nanograms per milliliter.

Statistical Analysis. Mixed-effects models in conjunction with permutation tests were used to test for differences in drug concentrations between compartments. These models were fit separately for each drug and used data from all subjects that had taken the drug. The intracellular drug concentrations were strongly positively skewed; hence, they were log transformed before model fitting. The model pooled data from all compartments unless otherwise stated and included indicator variables for each compartment to allow for testing for differences between blood and each compartment. When drug concentrations were found to be below the limit of detection, one-half of the limit of quantitation was imputed. Random intercepts were used to model the correlation of measurements arising from the same subject; however, such correlation was found to be rather low (with estimated intraclass correlations ranging from near 0 to 0.22). Parameter estimates were obtained by using restricted maximum likelihood; however, because the sample size was rather small for some drugs, hypothesis tests were conducted by using permutation tests. To use permutation testing in this context, we permuted measurements within each subject analogous to using permutation testing for testing in paired samples. We then compared the distribution of test statistics (e.g., the Wald statistic corresponding to the model coefficient associated with the indicator variable for LN) to the value that was observed. When time since initiating therapy was included in the model, there was evidence for lower concentrations of TFV ($P = 0.0246$) and FTC-TP ($P = 0.0026$) in all compartments at 6 mo.

Rates of decay (half-lives) were also estimated by using mixed-effects models. Models were fit with the logarithm of the size of the follicular dendritic cell network (FDCn) pool of virions as the response and time since initiating therapy as the explanatory variable in addition to random intercepts. The parameter estimates given by restricted maximum likelihood were then transformed to obtain point estimates of the mean half-lives.

A similar strategy was used to investigate associations between intracellular drug concentrations and the size of the FDCn pool of virus. First, data were pooled from all compartments other than blood (there is no FDCn pool in blood), and random intercept mixed-effects models were fit with the logarithm of the size of the FDCn pool as response and with indicators for compartment, the logarithm of drug concentration, and time since starting therapy as explanatory variables. These analyses found statistically significant negative associations between TFV-DP and FTC-TP concentrations and the size of the FDCn pool. A similar strategy found a negative association between the size of the viral RNA^+ cell population and TFV-DP concentration. Then the LN data were considered in isolation, and similar random intercept models were fit that allowed for the logarithm of the size of FDCn pool to depend on the logarithm of the drug concentrations, time since initiating therapy, and the interaction between these two terms. These models found statistically significant interactions between time and drug concentrations for TFV-DP and EFV, indicating that the half-life of virions in the FDCn pool is longer when drug concentration is lower in the LN.

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