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Nucleotide Analogue Prodrug Tenofovir Disoproxil Enhances Lymphoid Cell Loading following Oral Administration in Monkeys

Lucie Durand-Gasselin,[†] Koen K. A. Van Rompay,[‡] Jennifer E. Vela,[†] Ilana N. Henne,[†] William A. Lee,[†] Gerry R. Rhodes,[†] and Adrian S. Ray*,[†]

Gilead Sciences, Inc., Foster City, California 94404, and California National Primate Research Center, University of California, Davis, California 95616

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Abstract: The antiviral drug tenofovir (TFV) is orally administered as the fumarate salt of its disoproxil prodrug (TFV disoproxil fumarate (TDF)). TFV is a dianion at physiological pH and, as a result, has poor lipid membrane permeability. Administration of the lipophilic and cell-permeable prodrug, TFV disoproxil, enhances the oral absorption of TFV. In order to determine whether oral administration of TDF also increases distribution to sites of viral infection, the plasma and circulating lymphoid cell pharmacokinetics of TFV and its phosphorylated metabolites were assessed following a single oral TDF or subcutaneous TFV administration at doses yielding equivalent plasma exposures to TFV in macaques. Despite TFV disoproxil's lack of plasma stability and undetectable levels in the first plasma samples taken, oral administration of TDF resulted in 7.9-fold higher peripheral blood mononuclear cell exposures to the active metabolite, TFV-diphosphate. The apparent plasma terminal half-life ($t_{1/2}$) of TFV was also longer following oral TDF relative to subcutaneous TFV administration (median $t_{1/2}$ of 15.3 and 3.9 h, respectively), suggesting broader distribution to cells and tissues outside of the central plasma compartment. In conclusion, the disoproxil pro-moiety enhances not only the oral absorption of TFV but also tissue and lymphoid cell loading. These results illustrate that administration of even a fleeting prodrug can increase target tissue loading and give valuable insight for future prodrug development.

Keywords: HIV; prodrug; nucleotide; tenofovir

Introduction

Tenofovir (TFV) is widely used in the treatment of human immunodeficiency virus (HIV) infection and has recently received regulatory approval for hepatitis B virus therapy. TFV is orally administered as the fumarate salt of its lipophilic and cell-permeable prodrug, TFV disoproxil (TDF; Figure 1). TFV is an acyclic nucleotide analogue of 2′-deoxyadenosine monophosphate (dAMP).

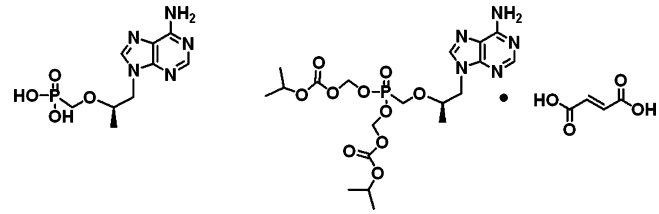

Figure 1. Structure of TFV and the fumarate salt of its orally bioavailable disoproxil prodrug (TDF).

Following two phosphorylation steps, TFV-diphosphate (DP) competes with endogenous 2′-deoxyadenosine triphosphate (dATP) for incorporation by virally encoded reverse transcriptases. Once incorporated, TFV serves as an obligate chain terminator of elongating viral DNA.

* To whom correspondence should be addressed. Mailing address: Gilead Sciences, Inc. 333 Lakeside Dr., Foster City, CA 94404. Phone: (650) 522-5536. Fax: (650) 522-1892. E-mail: adrian.ray@gilead.com.

[†] Gilead Sciences, Inc.

[‡] University of California, Davis.

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TFV disoproxil has >500-fold greater anti-HIV activity than TFV *in vitro*.[1] However, TFV disoproxil is not observed in plasma at the earliest collected time points following administration to patients[2,3] and, therefore, might be assumed to not affect the loading of lymphoid cells and tissues with the active metabolite, TFV-DP. TFV disoproxil has poor stability in human plasma with a reported half-life of less than 1 min.[4] The lack of detectable TFV disoproxil in plasma may also be related to degradation during intestinal absorption, where it is affected by intestinal efflux transport and hydrolase activity.[5,6]

In order to determine whether administration of TDF has an effect on TFV distribution, we investigated the plasma pharmacokinetic profile of TFV and intracellular levels of TFV and its phosphorylated metabolites in peripheral blood mononuclear cells (PBMCs) following single administration of subcutaneous TFV or oral TDF at doses resulting in equivalent plasma exposures to TFV in macaques. The findings reported here illustrate that oral administration of TDF results in efficient delivery of TFV into the systemic circulation and increases the loading of lymphoid cells with TFV-DP.

Experimental Section

Materials. TDF, TFV, TFV-MP, and TFV-DP were provided by Gilead Sciences, Inc. (Foster City, CA). Stable isotope-labeled [$^{13}C^{15}N$]2′-deoxyguanosine-TP (dGTP) and dATP were purchased from Spectra Stable Isotopes (Andover, MA). The analytical internal standards adefovir, GS-9148 (a nucleotide analogue with the chemical name [5-(6-amino-purin-9-yl)-4-fluoro-2,5-dihydro-furan-2-yloxymethyl)phosphonic acid]) and GS-9148-MP were obtained from Gilead Sciences, Inc., and 2-chloro-adenosine-TP (Cl-ATP) was obtained from Sigma-Aldrich (St. Louis, MO). All other reagents were the highest grade available from Sigma-Aldrich.

Animals. Rhesus macaques (*Macaca mulatta*) from the type D retrovirus-free and simian T-cell-lymphotropic virus type 1-free colony at the California National Primate Research Center (CNPRC) were used in these studies. The animals were housed in accordance with the standards of the American Association for Accreditation of Laboratory Animal Care and were receiving a standard commercial primate diet. Animals were handled in strict accordance with the Guide for the Care and Use of Laboratory Animals,[7] and the protocol was reviewed by the Institutional Animal Care and Use Committee (IACUC) at the University of California at Davis. The animals had previously been infected with nonpathogenic SIVmac1A11 (as described previously)[8] and were aviremic and healthy. Animals were between 17 and 25 months of age (3−4.3 kg) at the time of the initial pharmacokinetic experiments (aimed at determining doses resulting in equivalent plasma exposures) and approximately 40−43 months of age (4.2−8 kg) during the second and definitive experiment. For the drug administrations and blood collections, animals were immobilized with ketamine HCl (Parke-Davis, Morris Plains, NJ) at 10 mg/kg injected intramuscularly.

Drug Administration. For subcutaneous administration, TFV powder (isolated as the free base) was suspended in distilled water, dissolved by adding approximately 2 equiv of NaOH, volume adjusted and buffered with RPMI-1640 media to a final concentration of 60 mg/mL, and filter sterilized (0.2 μm filter; Nalgene, Rochester, NY). TFV solution was administered at final doses of 2.5−10 mg/kg subcutaneously into the backs of the monkeys. For oral administration, TDF powder was dissolved in RPMI-1640 media at a concentration of 8 mg/mL and was administered via orogastric intubation at doses of 20−30 mg/kg body weight.

Plasma and PBMC Sample Collection. Blood (approximately 3 mL) was collected by venipuncture at 0, 0.5, 1, 2, 4, 8, and 24 h postdose into Vacutainer tubes containing EDTA as an anticoagulant (BD Biosciences, San Jose, CA). Plasma was separated by spinning samples for 10 min at 900 × *g*. Supernatants were stored at −80 °C until further processing and analysis. At select time points (2, 4, 8, and 24 h postdose), 8−10 mL of blood was collected for PBMC

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isolation. Following separation of plasma, cells were isolated by Ficoll gradient separation (lymphocyte separation medium; MP Biomedicals, Aurora, OH) and washed in 4 °C 0.9% NaCl solution. Use of phosphate-buffered saline was avoided during sample preparation due to negative effects on analysis from its components. Isolated cells were washed with an NH_4Cl solution to remove contaminating red blood cells as described previously.[9] Isolated cell pellets (typically containing approximately 14 million PBMCs) were immediately frozen at −70 °C until further processing and analysis.

Liquid Chromatography Coupled to Tandem Mass Spectrometry (LC-MS/MS) Instrumentation. LC methods used an HTS PAL autosampler with cooled sample storage stacks set at 6 °C (Leap Technologies, Carrboro, NC) and an LC-20AD ternary pump system (Shimadzu Scientific Instruments, Columbia, MD). All LC-MS/MS analyses were done in positive ion and multiple reaction monitoring modes using a Sciex API-4000 mass spectrometer (Applied Biosystems, Foster City, CA).

Quantification of Plasma Concentrations of TFV. Plasma samples were subject to protein precipitation by addition of acetonitrile and formic acid to final concentrations of 66% and 0.2%, respectively. The structurally related nucleotide analogue adefovir was added as an internal standard. Samples were analyzed by a validated LC-MS/MS method. Analytes were separated using a 4 μm, 75 mm × 2 mm Synergi Hydro column (Phynomenex, Torrance, CA) using 0.2% formic acid and a linear gradient from 1% to 90% acetonitrile at a flow rate of 250 μL/min over 4.5 min. Six-point standard curves prepared in blank plasma covered concentrations of 3 orders of magnitude and showed linearity in excess of an r^2 value of 0.98. The lower limit of TFV quantification in plasma was 2.6 nM. Separately prepared quality control samples of 10, 80, and 4000 nM were analyzed at the beginning and end of each sample set to ensure accuracy and precision within 15%.

PBMC Sample Preparation. Frozen PBMC pellets had 2.6 pmol of the internal standards GS-9148 (used for TFV), GS-9148-MP (used for TFV-MP), and Cl-ATP (used for TFV-DP, dATP, and dGTP) added. Lysis of cell pellets was achieved by vortexing in 1 mL of ice cold 70% methanol. Cellular debris was removed by spinning at 15 000 × g, and the pellet was kept to evaluate the number of PBMCs using a previously described DNA-based biochemical assay using a standard curve generated from manually counted macaque PBMCs.[10] Supernatants were dried at 37 °C under nitrogen flow (Turbovap evaporator, Zymark) and resuspended in 50 μL of 1 mM phosphate buffer. A final spin (15 000 × g, 5 min) was used to remove any remaining particulate matter from extracts before injection on the LC-MS/MS system.

Simultaneous Quantifiaction of PBMC Concentrations of TFV-MP, TFV-DP, dATP, and dGTP. Elements of prior LC/MS/MS ion pairing nucleotide detection methods were combined including the use of 1,5-dimethylhexylamine as an ion pairing agent[11] and low flow LC coupled with positive ion mode MS/MS detection.[12] Briefly, analytes were separated using a 1.7 μm, 2.5 mm × 50 mm Acquity BEH column with a VanGaurd precolumn (Waters Corporation, Milford, MA) using an ion pairing buffer containing 10 mM 1,5-dimethylhexylamine and 3 mM ammonium formate and a multistage linear gradient from 5% to 75% acetonitrile at a flow rate of 100 μL/min over 10 min. Six-point standard curves prepared in blank PBMC matrices covered concentrations of 3 orders of magnitude and showed linearity for each analyte in excess of an r^2 value of 0.98. Quantification of endogenous dATP and dGTP was accomplished using standard curves of stable isotopically labeled nucleotides in cellular matrices as previously described.[12,13] The lower limits of quantification were between 38 and 66 fmol/sample for all analytes. Separately prepared quality control samples of 100 to 30 000 fmol/sample were analyzed at the beginning and end of each sample set to ensure accuracy and precision within 25%. Results in femtomoles per sample were then divided by the number of PBMCs; the extract for each injection was derived from, on average, 2−3 million cells, to obtain concentrations in fmol/10^6 PBMCs. Intracellular concentrations were then calculated assuming a mean cell volume of 0.2 μL/million PBMCs.[14]

Quantification of PBMC Concentrations of TFV. The presence of chromatographic interference in the method used for PBMC analyses of nucleotides described above did not allow for simultaneous quantification of TFV levels in PBMC. Therefore, all PBMC samples were subject to a second analysis to determine TFV levels using a similar LC-MS/MS methodology as that used for plasma (see above). Five point standard curves prepared in blank PBMC matrices showed linearity in excess of an r^2 value of 0.99. The lower limit of quantification was 153 fmol/sample.

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Table 1. Plasma TFV Pharmacokinetic Parameters following a Single Administration of 4 mg/kg Subcutaneous TFV or 30 mg/kg Oral TDF to Macaques

parameter	4 mg/kg subcutaneous TFV[a]	30 mg/kg oral TDF[a]	ratio[b]
AUC_{0-24} (nM·h)	24200 (16100−28300)	19900 (14400−32900)	0.8
$AUC_{0-\infty}$ (nM·h)	24200 (16200−28400)	25700 (21900−65900)[d]	1.1
C_{max} (nM)	13000 (10900−16300)	1580 (1280−2300)	0.12[c]
C_{24} (nM)	7.92 (7.50−8.49)	451 (292−1110)	57[c]
$t_{1/2}$ (h)	3.9 (3.4−4.3)	15.3 (11.7−20.6)[d]	3.9[c]

[a] Values represent the median (minimum−maximum) of $n = 5$ animals in each dosing arm unless noted otherwise. [b] Value for oral TDF divided by subcutaneous TFV. [c] Statistically significant as assessed by the nonparametric Mann−Whitney U-test; $P < 0.01$. [d] Values represent the median (minimum−maximum) of $n = 4$ animals. One animal was excluded from analyses due to a poorly defined terminal phase.

Data Analyses. Noncompartmental pharmacokinetic parameters were calculated using WinNonLin 5.01 (Pharsight Corporation, Mountain View, CA). Inter- and intraarm statistical comparisons were made using the nonparametric Mann−Whitney U-test. Statistical calculations were done using GraphPad Prism 4 (GraphPad Prism, Inc., La Jolla, CA). *P*-values less than or equal to 0.05 were considered significant.

Results

Determination of Doses Resulting in Equivalent Plasma TFV Exposures. Initial pharmacokinetic studies were done at oral TDF doses of 20 and 30 mg/kg (9.1 and 13.6 mg TFV equivalents per kg, respectively) and subcutaneous TFV doses of 2.5 and 10 mg/kg to allow for the estimation of doses resulting in equivalent plasma exposures to TFV (two to three monkeys per dose). Based on the observation of mg/kg dose normalized exposures approximately 8-fold higher following subcutaneous TFV relative to oral TDF administration (as determined by the area under the concentration versus time curve (AUC); data not shown), subsequent studies were done at doses of 30 mg/kg oral TDF and 4 mg/kg subcutaneous TFV. The 30 mg/kg oral TDF dose is approximately 2-fold higher than the surface area adjusted human dose of 300 mg used clinically but allowed for accurate detection of PBMC levels of TFV and its phosphorylated metabolites at the corresponding subcutaneous dose.

Plasma Pharmacokinetics following Oral TDF and Subcutaneous TFV. A sequential study design with a 6 week wash out period and five macaques per arm was chosen to compare the plasma and PBMC pharmacokinetics following a single administration of 30 mg/kg oral TDF or 4 mg/kg subcutaneous TFV. The noncompartmental plasma pharmacokinetic parameters are summarized in Table 1. As estimated from the initial studies summarized above, TFV plasma exposures over 24 h (AUC_{0-24}) and exposures extrapolated to infinity ($AUC_{0-\infty}$) did not significantly differ between the oral TDF and subcutaneous TFV study arms. Dose proportional changes in plasma exposures were observed relative to the initial studies completed to determine doses resulting in equivalent plasma exposures in both the oral TDF and subcutaneous TFV study arms (data not shown). However, marked differences in the plasma pharmacokinetic profile of TFV administered by the two routes were observed (Figure 2). Most notably, the terminal plasma half-life ($t_{1/2}$) of TFV was significantly longer following oral TDF relative to subcutaneous TFV administration. The increased persistence of plasma TFV in the oral TDF arm resulted in a 57-fold higher median TFV concentration measured at 24 h (C_{24}) despite an 8.2-fold lower median maximal plasma TFV concentration (C_{max}) relative to the subcutaneous TFV arm. Assuming complete bioavailability of the subcutaneous TFV dose, the relative $AUC_{0-\infty}$ values led to the calculation of an oral bioavailability of approximately 31% for TFV following oral administration of TDF. Due to plasma instability, a validated method for TFV disoproxil could not be obtained, and TFV disoproxil was not detected in plasma taken at any time. Consistent with previously reported findings in dogs,[15] low levels of the monosubstituted, intermediate metabolite of TFV disoproxil were observed in plasma from the oral treatment arm (AUC_{0-24} less than 0.3% that of plasma TFV).

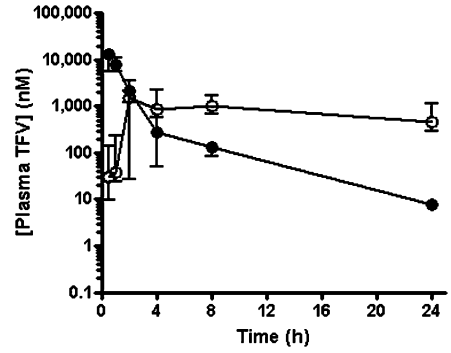

Figure 2. Pharmacokinetic profiles of TFV in plasma following a single administration of either 4 mg/kg subcutaneous TFV (●) or 30 mg/kg oral TDF (○) to macaques. Values represent the median (minimum−maximum) of five monkeys dosed with each regimen in a sequential study design with a 6 week washout period.

PBMC Pharmacokinetics. In contrast to the equivalent plasma TFV exposures observed, intracellular TFV, TFV-MP, and TFV-DP AUC_{0-24} in PBMCs were significantly higher following oral TDF relative to subcutaneous TFV

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Table 2. PBMC AUC_{0-24} to TFV, TFV-MP, and TFV-DP following a Single Administration of 4 mg/kg Subcutaneous TFV or 30 mg/kg Oral TDF to Macaques

	intracellular AUC_{0-24} (nM·h)		
nucleotide	4 mg/kg subcutaneous TFV[a]	30 mg/kg oral TDF[a]	ratio[b]
TFV	2800 (1110−3200)	10200 (5420−13700)	4.9[c]
TFV-MP	1220 (384−2990)	6630 (5540−11100)	5.4[c]
TFV-DP	4650 (1910−9150)	36600 (24500−44400)	7.9[c]

[a] Values represent the median (minimum−maximum) of $n = 5$ animals in each dosing arm. [b] Value for oral TDF divided by subcutaneous TFV. [c] Statistically significant as assessed by the nonparametric Mann−Whitney U-test; $P < 0.01$).

administration (Table 2). Intracellular TFV, TFV-MP, and TFV-DP levels were consistently greater at all time points in the oral TDF arm (Figure 3). For example, the median C_{max} values of TFV-DP in PBMC were 2200 and 323 nM in the oral TDF and subcutaneous TFV arms, respectively ($P = 0.0079$). TFV-DP rapidly accumulated in PBMCs and was found to persist with a $t_{1/2}$ of >24 h in both arms. Dose-proportional changes in PBMC exposures to TFV and its phosphorylated metabolites were observed relative to the initial studies completed to determine doses resulting in equivalent plasma exposures in both the oral TDF and subcutaneous TFV study arms (data not shown).

Levels of Endogenous 2′-Deoxynucleotides. Levels of dATP and dGTP in PBMCs were determined to further validate cell collection techniques, the consistency of PBMC collection between the two arms of the study, and the effectiveness of the cell lysis procedure and to ensure that no dephosphorylation was occurring during sample preparation. Similar levels of dATP and dGTP were observed between arms and over time in the same arm (Figure 4). Determined concentrations of dATP and dGTP (in the 1−5 μM range) were similar to those measured previously in lymphoid cells by different methodologies.[16,17]

Discussion

Development of the acyclic nucleoside phosphonate TFV as an oral antiviral agent was inextricably linked to identification of the disoproxil prodrug required to increase this charged nucleotide analogue's oral bioavailability.[18] In the present study, oral administration of TDF resulted in effective delivery of TFV into the systemic circulation in macaques.

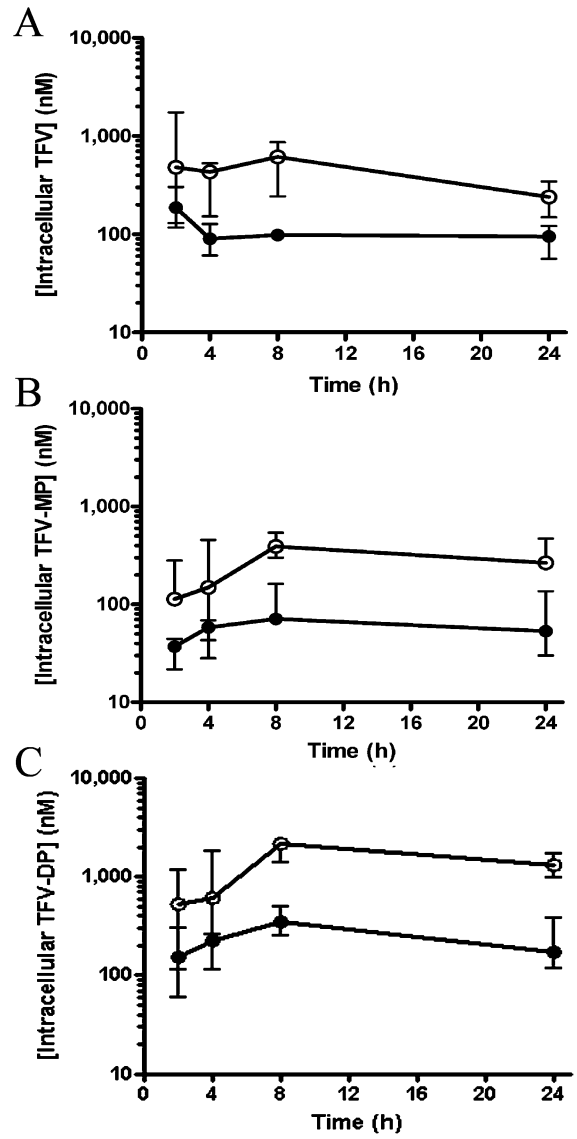

Figure 3. Pharmacokinetic profiles of TFV (A), TFV-MP (B), and TFV-DP (C) in PBMCs following a single administration of either 4 mg/kg subcutaneous TFV (●) or 30 mg/kg oral TDF (○) to macaques. Values represent the median (minimum−maximum) of five monkeys dosed with each regimen in a sequential study design with a 6 week washout period.

The oral bioavailability observed in macaques here (31%) is similar to that reported in humans (25%).[3] Consistent with a higher surface area adjusted dose relative to the human clinical dose, the 30 mg/kg oral TDF dose in macaques resulted in plasma TFV $AUC_{0-\infty}$ approximately 3-fold higher than the steady-state AUC_{0-24} observed in humans following repeat dosing at 300 mg once a day (29000 and 9510 nM·h, respectively).[3]

Consistent with the higher plasma exposures observed following oral administration of 30 mg/kg TDF to macaques relative to administration of 300 mg to patients, approximately 4-fold higher intracellular TFV-DP levels were observed here (2200 nM) relative to those observed in PBMCs from HIV-infected patients treated with TDF (ap-

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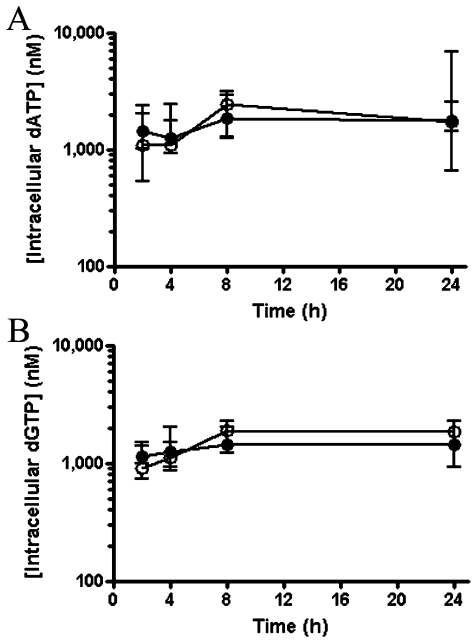

Figure 4. Intracellular levels of the endogenous nucleotides dATP (A) and dGTP (B) in PBMCs following administration of either 4 mg/kg subcutaneous TFV (●) or 30 mg/kg oral TDF (○) to macaques. Values represent the median (minimum−maximum) of five monkeys dosed once with each regimen in a sequential study design with a 6 week washout period.

proximately 500 nM).[11,19] Despite the lack of a significant difference in plasma TFV exposures between arms, intracellular levels of TFV, TFV-MP, and TFV-DP were markedly increased following oral TDF relative to subcutaneous TFV administration. This contrasted with the endogenous nucleotides dATP and dGTP, both found to have similar levels between the two arms. In contrast to the oral TDF arm, in the subcutaneous TFV arm, the ratio of PBMC TFV-DP to plasma TFV was lower than what has been observed in patients orally administered TDF. Combined, these results suggest that oral administration of TDF to humans causes similar increases in PBMC loading as what has been observed in this study in macaques. Enhanced TFV delivery to PBMCs after oral TDF administration has also been observed in beagle dogs.[18] Because PBMCs are circulating lymphocytes and represent a compartment infected by HIV, the increased level of the active metabolite, TFV-DP, in these cells following oral TDF administration has important implication for clinical efficacy. Indeed, a cross-study comparison of short-term clinical efficacy studies comparing intravenous TFV and oral TDF suggests higher efficacy following oral TDF administration.[2,20] While the concentrations of intracellular TFV and its phosphorylated metabolites differed significantly, levels persisted over the 24 h sampling period in both arms. Similarly, long TFV-DP half-lives have been observed in various cell types *in vitro*[1,21] and in PBMCs taken from patients *in vivo*.[11,19]

Following subcutaneous administration, TFV was observed to have a relatively short $t_{1/2}$ (3.9 h) and a limited steady-state volume of distribution (V_{ss}, 0.75 L/kg). This calculated V_{ss} likely represents a slight overestimate, because it assumes immediate and complete bioavailability of the subcutaneous dose. Nevertheless, the V_{ss} estimated here leads to a similar conclusion as that made based on intravenous administration in humans[3] and suggests limited distribution outside of total body water (total body water is approximately 0.7 L/kg in monkeys).[22] TFV is not catabolized and is cleared from plasma exclusively through renal elimination by the combined action of glomerular filtration and active tubular secretion.[23] Consistent with studies in other species, in macaques the clearance of TFV (0.60 L/h/kg) exceeded the glomerular filtration rate and approached renal blood flow (in monkeys, approximately 0.13 and 1.66 L/h/kg, respectively).[22] In addition to enabling effective oral delivery of TFV into plasma and PBMCs, the plasma pharmacokinetic profile for TFV following oral administration of TDF suggests that TDF administration also resulted in a wider general distribution of TFV outside of the central plasma compartment. Since plasma $t_{1/2}$ is a function of volume and clearance and the clearance of TFV is unlikely route dependent, the marked increase in the plasma $t_{1/2}$ of TFV observed after oral TDF administration likely reflects the prodrug more widely distributing TFV and its phosphorylated metabolites to cells and tissues and slow release of TFV back into the central plasma compartment where it is subject to renal elimination.

Taken together, the plasma and PBMC profiles suggest that two factors contribute to the more efficient PBMC loading observed in the oral TDF arm. First, TDF directly loads PBMCs immediately following administration while it is still present in plasma. Direct PBMC loading by TDF is illustrated by the higher TFV and phosphorylated me-

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tabolite levels observed in PBMCs during the first 2 h in the oral TDF arm despite plasma exposures to TFV greater than 10-fold lower than the subcutaneous TFV arm (median AUC_{0-2} of 842 and 18900 nM·h, respectively). Second, the wider distribution of TFV resulting from administration of TDF indirectly facilitates continued PBMC loading due to the longer duration of plasma exposures to TFV. Plasma levels of TFV in the oral TDF arm exceeded the subcutaneous TFV arm following the 2 h sampling. Combined, these two processes serve to explain the consistently higher intracellular TFV and phosphorylated metabolite levels over the entire dosing interval following oral TDF administration.

In summary, our study quantitatively shows that the addition of the disoproxil pro-moiety not only allows for effective oral delivery of TFV into the systemic circulation but also enhances loading of TFV-DP into PBMCs. In addition to adding valuable insight into the pharmacology of TDF, understanding the effect of pro-moieties on target tissue loading will help in the development of agents with improved efficacy. We have studied next generation phosphonoamidate prodrugs that exhibit improved stability, measurable prodrug levels in the systemic circulation, and further enhanced target cell and tissue loading,[4,24−26] and others have applied phosphate prodrugs to nucleoside analogues to improve pharmacologic activity.[27−29]

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