Pharmacokinetics of tenofovir monoester and association with intracellular tenofovir diphosphate following single-dose tenofovir disoproxil fumarate

Kristina M. Brooks [1], Mustafa E. Ibrahim[1], Jose R. Castillo-Mancilla[2], Samantha MaWhinney[3],
Keisha Alexander[1], Scott Tilden[1], Becky Jo Kerr[1], Lucas Ellison[1], Cricket McHugh[1], Lane R. Bushman[1],
Jennifer J. Kiser[1], Sybil Hosek[4], Gregory D. Huhn[4] and Peter L. Anderson[1]*

[1]Department of Pharmaceutical Sciences, Skaggs School of Pharmacy and Pharmaceutical Sciences, University of Colorado Anschutz
Medical Campus (AMC), Aurora, CO, USA; [2]Department of Medicine, School of Medicine, University of Colorado AMC, Aurora, CO, USA;
[3]Department of Biostatistics and Informatics, Colorado School of Public Health, University of Colorado AMC, Aurora, CO, USA;
[4]Department of Medicine, Stroger Hospital of Cook County, Chicago, IL, USA

*Corresponding author. University of Colorado Skaggs School of Pharmacy and Pharmaceutical Sciences, 12850 E Montview Blvd, V20-C238, Aurora,
CO 80045, USA. Tel: +1 (303) 724-6128; Fax: +1 (303) 724-6135; E-mail: peter.anderson@ucdenver.edu

Background: Tenofovir monoester is a relatively lipophilic intermediate formed during the hydrolysis of tenofovir disoproxil to tenofovir. Its clinical pharmacokinetic profile and influence on the cellular pharmacology of tenofovir diphosphate have not been reported.

Methods: Plasma, PBMC and dried blood spots (DBS) were obtained from HIV-uninfected adults participating in a randomized, cross-over bioequivalence study of single-dose tenofovir disoproxil fumarate (TDF)/emtricitabine unencapsulated or encapsulated with a Proteus® ingestible sensor. Plasma pharmacokinetics of tenofovir monoester and tenofovir were characterized using non-compartmental methods. Relationships with tenofovir diphosphate in DBS and PBMC were examined using mixed-effects models.

Results: Samples were available from 24 participants (13 female; 19 white, 3 black, 2 Hispanic). Tenofovir monoester appeared rapidly with a median (range) T_{max} of 0.5 h (0.25–2) followed by a rapid monophasic decline with a geometric mean (coefficient of variation) $t_{1/2}$ of 26 min (31.0%). Tenofovir monoester C_{max} was 131.6 ng/mL (69.8%) and AUC_{0-4} was 93.3 ng·h/mL (47.9%). The corresponding values for plasma tenofovir were 222.2 ng/mL (37.1%) and 448.1 ng·h/mL (30.0%). Tenofovir monoester $AUC_{0-\infty}$ (but not tenofovir $AUC_{0-\infty}$) was a significant predictor of tenofovir diphosphate in both PBMC ($P=0.015$) and DBS ($P=0.005$), increasing by 3.8% (95% CI 0.8%–6.8%) and 4.3% (95% CI 1.5%–7.2%), respectively, for every 10 ng·h/mL increase in tenofovir monoester.

Conclusions: Tenofovir monoester C_{max} and AUC_{0-4} were 59.2% and 20.6% of corresponding plasma tenofovir concentrations. Tenofovir monoester was significantly associated with intracellular tenofovir diphosphate concentrations in PBMC and DBS, whereas tenofovir concentrations were not. Tenofovir monoester likely facilitates cell loading, thereby increasing tenofovir diphosphate exposures *in vivo*.

Introduction

Tenofovir is an NRTI used in the treatment and prevention of HIV infection. Tenofovir exerts its mechanism of action following conversion to its active diphosphate form within cells, competing with endogenous deoxyadenosine triphosphate and ultimately terminating chain elongation during viral replication.[1] Tenofovir is hydrophilic and permeates poorly across the intestinal tract and other cell membranes,[2,3] and thus was formulated as a prodrug to improve its oral absorption and cell permeability, first as tenofovir

disoproxil fumarate (TDF) and more recently as tenofovir alafenamide fumarate (TAF). Entry of tenofovir into target lymphoid cells is critical to its efficacy.

Multiple studies have demonstrated the enhanced ability of the prodrug form to load cells in comparison with tenofovir. In monkeys, oral administration of TDF resulted in ~8-fold higher tenofovir diphosphate concentrations in lymphoid cells as compared with an equivalent subcutaneous tenofovir dose.[4] That study suggested that some tenofovir disoproxil (or monoester) reached the

systemic circulation to facilitate cell loading. In comparison with TDF, and despite lower tenofovir dose equivalents, TAF results in ~2.4- to 7-fold higher tenofovir diphosphate concentrations.[5–7] This demonstrates more efficient cell loading for TAF, due in part to its superior stability and longer half-life in blood of 25 min[5] versus <5 min for TDF.[8] The pathways for conversion of the prodrug moieties have been described in the literature. However, the role and pharmacokinetics (PK) of intermediates formed during the conversion of these prodrugs remain unknown.

Following oral administration, tenofovir disoproxil is hydrolysed by esterases in the intestinal tract, liver and blood to tenofovir monoester and then tenofovir.[9] When tenofovir is present in cells, it undergoes phosphorylation by intracellular kinases to its active moiety, tenofovir diphosphate.[1] Previous literature has reported that the conversion from TDF to tenofovir occurs rapidly, resulting in tenofovir as the only circulating moiety in blood.[1,10,11] However, more recently, Nye et al.[12] reported the detection of tenofovir monoester in plasma during the course of assay development, which circulated at low levels following TDF administration. The plasma PK of tenofovir monoester has not been characterized in vivo, but physiochemical characteristics of tenofovir monoester and in vitro studies suggest that this moiety can penetrate cells more efficiently than tenofovir. Predicted octanol–water partition (log P) coefficients for tenofovir monoester and tenofovir are −0.1 and −1.6, respectively (Figure 1).[13,14] Though the disoproxil form is the most lipophilic of the three,[15] tenofovir monoester is more lipophilic than tenofovir and thus could influence intracellular loading and tenofovir diphosphate concentrations.

Tenofovir diphosphate is the active form of tenofovir and is quantifiable in multiple cell and tissue types. Several studies have focused on the concentrations of this moiety in PBMC, given that HIV infects these cell types (notably CD4 T lymphocytes). Tenofovir diphosphate is also quantifiable in red blood cells, which can be assayed using dried blood spots (DBS). Tenofovir diphosphate has a long $t_{1/2}$ in DBS (17 days), which has been used to examine cumulative medication adherence to TDF in persons living with HIV[16–18] and HIV-uninfected individuals on pre-exposure prophylaxis.[19–22] Tenofovir diphosphate exhibits coefficients of variation (CVs) of ~50% and ~30% in PBMC and DBS at steady-state, but sources of variability have been difficult to identify.[23,24] If tenofovir monoester contributes to cell loading in vivo, then variability in its PK could be a source of variability for intracellular tenofovir diphosphate.

In support of this possibility, we recently found that tenofovir diphosphate concentrations were elevated in persons on TDF in combination with sofosbuvir-containing therapies,[25] and this occurred concurrently with increases in tenofovir monoester concentrations in individuals on TDF in combination with ledipasvir/sofosbuvir.[26] However, the full PK profile and potential influence of tenofovir monoester on cell loading have not been adequately studied. Thus, the aims of the present study were to characterize the plasma PK of tenofovir monoester in humans following single-dose administration of TDF 300 mg/emtricitabine, and to examine relationships between tenofovir monoester and intracellular tenofovir diphosphate in PBMC and DBS.

Patients and methods

Study design

Samples were obtained from a previously conducted intensive PK study in HIV-uninfected individuals at low risk of HIV designed to determine the bioequivalence of TDF/emtricitabine at 300/200 mg when co-encapsulated with the Proteus® Sensor System (Proteus Digital Health®, Redwood City, CA, USA) (ClinicalTrials.gov NCT02968576).[27] Enrolment began in December 2016, with the last follow-up visit in May 2017. Secondary endpoints of the study included examining sources of PK variability in drug and anabolite concentrations in DBS and PBMC. Briefly, participants were randomized to receive single oral doses of unencapsulated or co-encapsulated TDF/emtricitabine, separated by a 14 day washout between study visits. PK assessments were performed following an overnight fast of at least 10 h at both study visits, with serial PK sampling at time 0 (pre-dose), 0.25, 0.5, 1, 2, 4, 6, 10, 24, 48 and 72 h post-dose.

Ethics

This study was approved by the Colorado Multiple Institutional Review Board (16–1478) and all participants provided written informed consent.

Sample preparation

Samples of interest included plasma, PBMC and DBS. Blood samples for plasma separation were collected in EDTA tubes at all specified timepoints
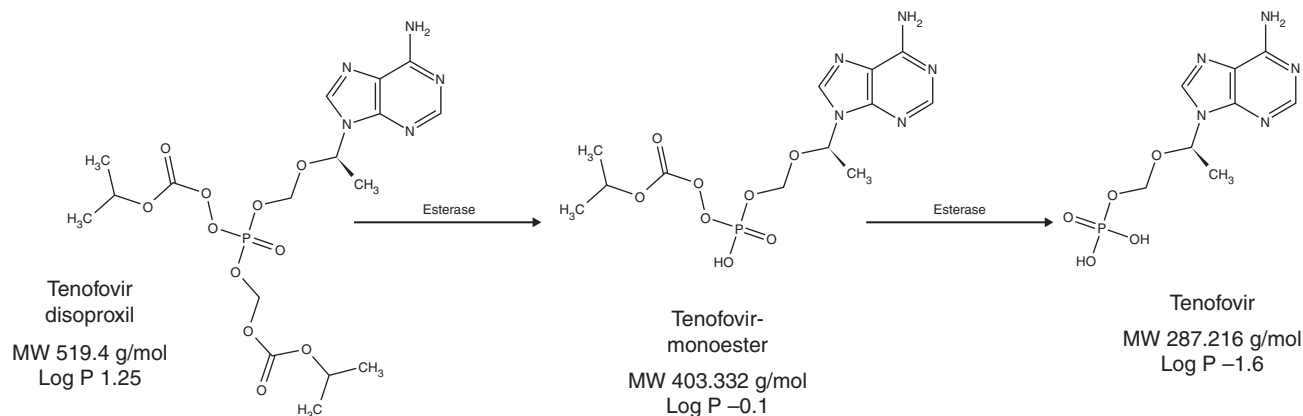
Figure 1. Structures of tenofovir disoproxil, tenofovir monoester, and tenofovir. Figure adapted from Murphy RA, Valentovic MA. Factors contributing to the antiviral effectiveness of tenofovir. *J Pharmacol Exp Ther* 2017; **363**: 156–163, by permission of The American Society for Pharmacology and Experimental Therapeutics.

above. Whole blood was spun down at 1200 **g** for 10 min at 4°C, and the plasma was transferred to aliquots and stored at −80°C until analysis. PBMC and DBS were isolated at the 24 h post-dose timepoint at both study visits. Sample processing procedures followed previously described methods[23,28] and samples were stored at −80°C until analysis.

Assay methods

Concentrations of tenofovir in plasma and tenofovir diphosphate in PBMC and DBS were quantified using validated LC-MS/MS methods as previously described.[29–31] Tenofovir diphosphate concentrations in PBMC were normalized to 10^6 cells and in DBS were normalized to a 3 mm punch. The lower limit of quantification (LLOQ) for tenofovir in plasma was 10 ng/mL and for tenofovir diphosphate in PBMC and DBS it was 5.0 and 25 fmol/sample, respectively. A validated punch stacking procedure was used when necessary to ensure quantifiable concentrations in DBS. A method for tenofovir disoproxil in plasma was not developed due to instability of the analyte in this matrix. A UPLC-MS-MS assay was developed for the determination of tenofovir monoester (Toronto Research Chemicals, Toronto, Ontario, Canada) in human plasma, and was validated for use with EDTA plasma matrix. Plasma proteins were precipitated with trifluoroacetic acid followed by chromatographic separation performed on a Waters Acquity H-Class UPLC. Separation was achieved with a Waters Acquity HSS T3 (2.1×100 mm, 1.8 µm) reversed-phase UPLC column with gradient elution at 0.5 mL/min. The gradient went from 2% acetonitrile to 50% acetonitrile (0.1% formic acid) over 3.0 min. Detection was achieved by electrospray ionization (ESI) positive ionization tandem MS on a Waters Xevo TQ-S Micro detector. Precursor/product transitions (*m/z*) in the positive ion mode were 404/176. Linearity of the method was in the range 0.1–500 ng/mL using a 1/concentration^2 weighted calibration curve. The assay had a minimum quantifiable limit of 0.1 ng/mL when 0.25 mL of plasma was analysed. Intra-day accuracy was within ±10.6% and precision ≤10.3%. The inter-assay accuracy was within ±6.8% and precision ≤7.9%. Carryover and matrix effects were insignificant for the EDTA plasma matrix. Conditional stability was shown for two freeze/thaw cycles, 6 h in matrix at ambient conditions, and extracted sample stability for 8 days.

PK analysis

PK parameters for tenofovir monoester and tenofovir in plasma were calculated via non-compartmental methods (Phoenix WinNonlin, v8.0). C_{max} and last measurable concentrations (C_{last}) were determined based on direct visualization of the data. The elimination rate constant (k_e) was estimated as the value of the linear regression slope of data points during the elimination phase. Half-life was calculated as $\ln(2)/k_e$. AUC_{0-4} and AUC_{0-24} were estimated using the linear up-log down trapezoidal rule. $AUC_{0-\infty}$ was determined by adding AUC through the last measurable timepoint to the value of the last measurable concentration divided by k_e. Results below the LLOQ were imputed as half the LLOQ for the primary analysis and as missing for the sensitivity analysis.

Statistical analysis

Statistical analyses were performed using SAS Enterprise Guide v7.1 (SAS Institute, Inc., Cary, NC, USA). Demographic variables were summarized as mean (SD). Plasma PK parameters were natural log (ln)-transformed prior to analysis and summarized as geometric mean (GM) and CV. Ratio comparisons between tenofovir monoester and tenofovir for key PK parameters were compared directly and following molar conversion of these moieties from ng/mL to nM to compare tenofovir equivalents using molecular weights of 403.332 and 287.216 g/mol, respectively.[13,14] Associations between tenofovir monoester and tenofovir in plasma were examined by linear regression.

The associations of tenofovir monoester and tenofovir with intracellular tenofovir diphosphate concentrations in PBMC and DBS were examined using mixed-effects models to account for repeated measures, arising from the cross-over design. Tenofovir monoester $AUC_{0-\infty}$ and tenofovir $AUC_{0-\infty}$ were analysed separately as fixed effects and participants as random effects. For the mixed-effects models, the primary outcome of interest (tenofovir diphosphate) was ln-transformed, and predictors of interest remained on the original scale. Clinical and demographic factors, including baseline BMI, estimated glomerular filtration rate (eGFR) and sex, in addition to potential confounding effects of formulation (unencapsulated versus encapsulated), randomization sequence (unencapsulated to encapsulated, or encapsulated to unencapsulated) and visit number, were also examined as predictors of tenofovir diphosphate concentrations. These factors were then examined as covariates in base models of tenofovir monoester $AUC_{0-\infty}$ and tenofovir $AUC_{0-\infty}$, and final model selection was based on improvement in the Akaike information criterion by at least 2 points from the base models. Model results are reported as percentage change (95% CI) for predictors of interest.

For illustrative purposes, raw geometric mean tenofovir monoester $AUC_{0-\infty}$ results from visit 1 were divided into quartiles and comparisons were made between the lowest and highest quartiles versus corresponding tenofovir diphosphate concentrations in PBMC and DBS using the first study visit. *P* values for these comparisons reflected two-tailed unpaired *t*-tests. These results were back-transformed to the original scale and presented as geometric mean (CV) for interpretability of study findings.

Results

Study population

A total of 24 participants were enrolled (13 female; 19 white, 3 black, 2 Hispanic) with data available from two study visits, resulting in 48 total observations for analysis. Mean (SD) age, weight and BMI were 28.1 (3.5) years, 74.5 (14.2) kg and 25.2 (4.0) kg/m^2, respectively. Mean (SD) eGFR was 118.7 (24.2) mL/min/1.73 m^2.

PK of tenofovir monoester versus tenofovir

This study was not designed to assess tenofovir monoester bioequivalence. Therefore, summary PK focused on the unencapsulated formulation. Tenofovir monoester concentrations were rapidly quantifiable following oral administration of TDF/emtricitabine and reached peak concentrations of 131.6 ng/mL at a median of 0.5 h post-dose (Figure 2a and Table 1). The concentration–time profile for this moiety exhibited monophasic decline, with a geometric mean $t_{1/2}$ of 0.44 h (26 min). By 4 h post-dose, 7/24 samples were below the LLOQ of 0.1 ng/mL for tenofovir monoester. Tenofovir monoester AUC_{0-4} was 93.3 ng·h/mL, which was >99% of the $AUC_{0-\infty}$ for this moiety. Tenofovir monoester C_{max} and AUC_{0-4} were 59.2% and 20.6% of tenofovir on a ng/mL basis (42.2% and 14.7% on a nM basis). Tenofovir monoester AUC_{0-4} demonstrated a significant relationship with tenofovir AUC_{0-4} [3.1% increase for every 10 ng·h/mL increase (95% CI 0.4%–5.8%), $R^2=0.20$, $P=0.03$] (Figure 2b), though this relationship weakened with tenofovir versus tenofovir monoester $AUC_{0-\infty}$ comparisons [2.3% increase for every 10 ng·h/mL increase (95% CI −0.5% to 5.1%), $R^2=0.12$, $P=0.10$] (data not shown).

Influence on tenofovir diphosphate concentrations

Results from both the encapsulated and unencapsulated forms were used for analyses throughout this section. As expected for a 2 week washout, visit was a significant predictor of tenofovir
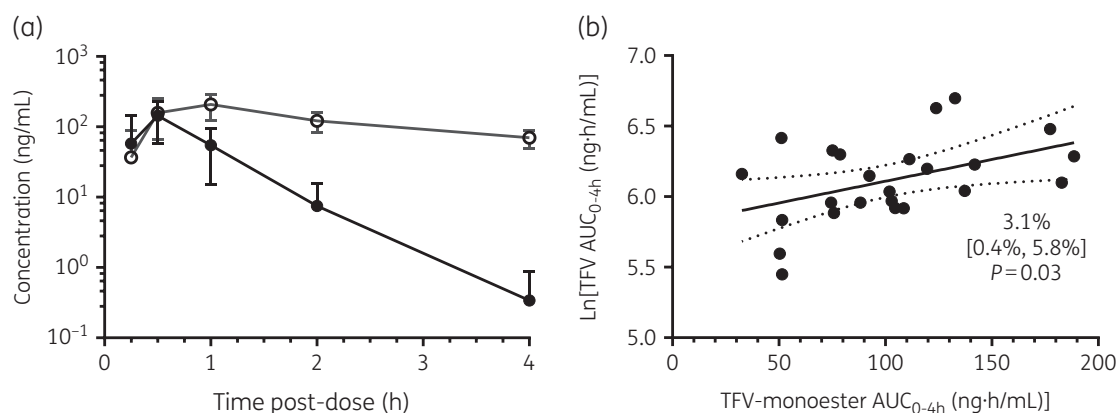
Figure 2. (a) Mean $\pm$ SD plasma concentrations of tenofovir monoester (filled circles) and tenofovir (TFV; open circles) until 4 h post-dose. (b) Tenofovir versus tenofovir monoester AUC_{0-4} in plasma. Tenofovir AUC_{0-4} was ln transformed prior to analysis for comparison with tenofovir monoester (original scale). Data are presented as line of best fit with 95% CI indicated by the dotted lines.

Table 1. Pharmacokinetics of tenofovir monoester and tenofovir (TFV) in plasma

Moiety	AUC_{0-4} (ng·h/mL)	$AUC_{0-\infty}$ (ng·h/mL)	T_{max} (h)	C_{max} (ng/mL)	C_4 (ng/mL)	$t_{1/2}$ (h)
TFV monoester[a]	93.3 (47.9%)	93.9 (46.8%)	0.5 (0.25–2.0)	131.6 (69.8%)	0.17 (152.4%)	0.44 (31.0%)
TFV[a]	448.1 (30.0%)	1986.0 (26.9%)	1.0 (0.5–2.0)	222.2 (37.1%)	67.4 (26.9%)	20.5 (24.6%)
TFV monoester versus TFV[b]	20.6% (17.2%–24.6%)	4.8% (3.9%–5.7%)	−0.5 (−1.5 to 0.0)	59.2% (49.0%–71.5%)	0.3% (0.2%–0.4%)	2.1% (1.8%–2.5%)

[a]Data are presented as geometric mean (CV), except T_{max}, which is reported as median (range).
[b]Comparisons between tenofovir monoester and tenofovir are presented as geometric mean ratio (95% CI), except T_{max}, which is reported as the median (range) difference.

diphosphate concentrations in DBS but not PBMC. Geometric mean (CV) tenofovir diphosphate concentrations in PBMC at 24 h post-dose were 10.8 (42.4%) and 10.3 (54.7%) fmol/10^6 cells at visits 1 and 2, respectively. In DBS, geometric mean tenofovir diphosphate concentrations were 45.3 (48.2%) and 88.3 (51.9%) fmol/punch at visits 1 and 2, respectively ($P<0.0001$) (Table 2). Formulation, sequence and clinical covariates of sex, BMI and eGFR were not significantly associated with tenofovir diphosphate concentrations in PBMC or DBS in univariate models (Table 2).

Tenofovir monoester was a significant predictor of tenofovir diphosphate concentrations in both PBMC ($P=0.015$) and DBS ($P=0.005$), the latter of which was controlled for study visit. For every 10 ng·h/mL increase in the $AUC_{0-\infty}$ of tenofovir monoester, concentrations of tenofovir diphosphate in PBMC increased by 3.8% (95% CI 0.8%–6.8%) (Table 2 and Figure 3) and 4.3% (95% CI 1.5%–7.2%) in DBS. For illustration, at visit 1 participants in the lowest quartile for tenofovir monoester $AUC_{0-\infty}$ had a geometric mean of 40.9 ng·h/mL, with corresponding tenofovir diphosphate concentrations of 9.4 fmol/10^6 cells in PBMC and 36.8 fmol/punch in DBS. In comparison, participants in the highest quartile for tenofovir monoester $AUC_{0-\infty}$ had a geometric mean of 167.9 ng·h/mL, and corresponding tenofovir diphosphate concentrations of 13.3 fmol/10^6 cells in PBMC ($P=0.10$) and 68.3 fmol/punch in DBS ($P=0.004$). In contrast to tenofovir-monoester, plasma tenofovir $AUC_{0-\infty}$ was not a significant predictor of tenofovir diphosphate concentrations in PBMC (95% CI −0.05% to 0.45%; $P=0.11$) or DBS (95% CI −0.27% to 0.27%; $P>0.99$). Point estimates, 95% CIs and

P values were similar for the sensitivity analysis with LLOQ values entered as missing for tenofovir monoester and tenofovir $AUC_{0-\infty}$.

Discussion

Tenofovir monoester exhibited significant systemic exposures in humans following oral administration of TDF/emtricitabine. Tenofovir monoester concentrations were ~42% and ~15% of tenofovir C_{max} and AUC_{0-4}, respectively, on an nM equivalent basis, and this moiety was rapidly eliminated with a $t_{1/2}$ of 0.44 h. In line with its increased lipophilicity compared with tenofovir, tenofovir monoester was a significant predictor of tenofovir diphosphate concentrations in PBMC and DBS, whereas no significant association was found for tenofovir. For every 10 ng·h/mL increase in tenofovir monoester $AUC_{0-\infty}$, tenofovir diphosphate increased by ~4% in both PBMC and DBS. These findings challenge previous literature dismissing the existence of tenofovir monoester in human plasma,[10,11,32] and reveal the potential clinical impact of this moiety on *in vivo* cell loading and tenofovir diphosphate concentrations, which are relevant to the achievement of protective and therapeutic concentrations.

The hydrolysis of TDF is primarily catalysed by carboxylesterases (CES),[9] but phosphodiesterases[9] and lipases have also been implicated in this pathway.[33] CES are found in several tissue types and multiple families have been identified, but CES1 and CES2 are the major forms found in humans.[34] CES1 is predominantly expressed in the liver, though CES2 is also found in low amounts.

Table 2. Univariate associations of tenofovir monoester, tenofovir and clinical covariates with tenofovir diphosphate concentrations in PBMC and DBS

Variable	TFV-DP in PBMC			TFV-DP in DBS[a]		
	change in TFV-DP	95% CI	P value[b]	change in TFV-DP	95% CI	P value[b]
Visit (2 versus 1)	−5.0%	−24.7% to 20.0%	0.66	95.1%	59.2%–139.0%	<**0.0001**
Randomization sequence	5.1%	−23.8% to 44.9%	0.75	25.0%	−11.0% to 75.6%	0.19
Formulation (encapsulated versus not)	−10.0%	−28.5% to 13.1%	0.35	−4.8%	−22.7% to 17.2%	0.63
Sex (female versus male)	−9.3%	−34.1% to 24.9%	0.53	−5.9%	−33.9% to 33.9%	0.73
BMI (kg/m^2)	−2.2%	−6.0% to 1.7%	0.25	−1.0%	−5.3% to 3.5%	0.63
eGFR (mL/min/1.73 m^2)	0.1%	−0.6% to 0.8%	0.84	−0.0%	−0.8% to 0.7%	0.97
Tenofovir monoester AUC$_{0-\infty}$ (per 10 ng·h/mL)[c]	3.8%	0.8%–6.8%	**0.015**	4.3%	1.5%–7.2%	**0.005**
Tenofovir AUC$_{0-\infty}$ (per 10 ng·h/mL)[c]	0.2%	−0.1% to 0.5%	0.11	0.0%	−0.3% to 0.3%	>0.99

TFV-DP, tenofovir diphosphate.
[a]Reported point estimates for TFV-DP in DBS were controlled for study visit.
[b]P values <0.05 are shown in bold.
[c]Percentage changes reported per 10 ng·h/mL increase in tenofovir monoester or tenofovir.
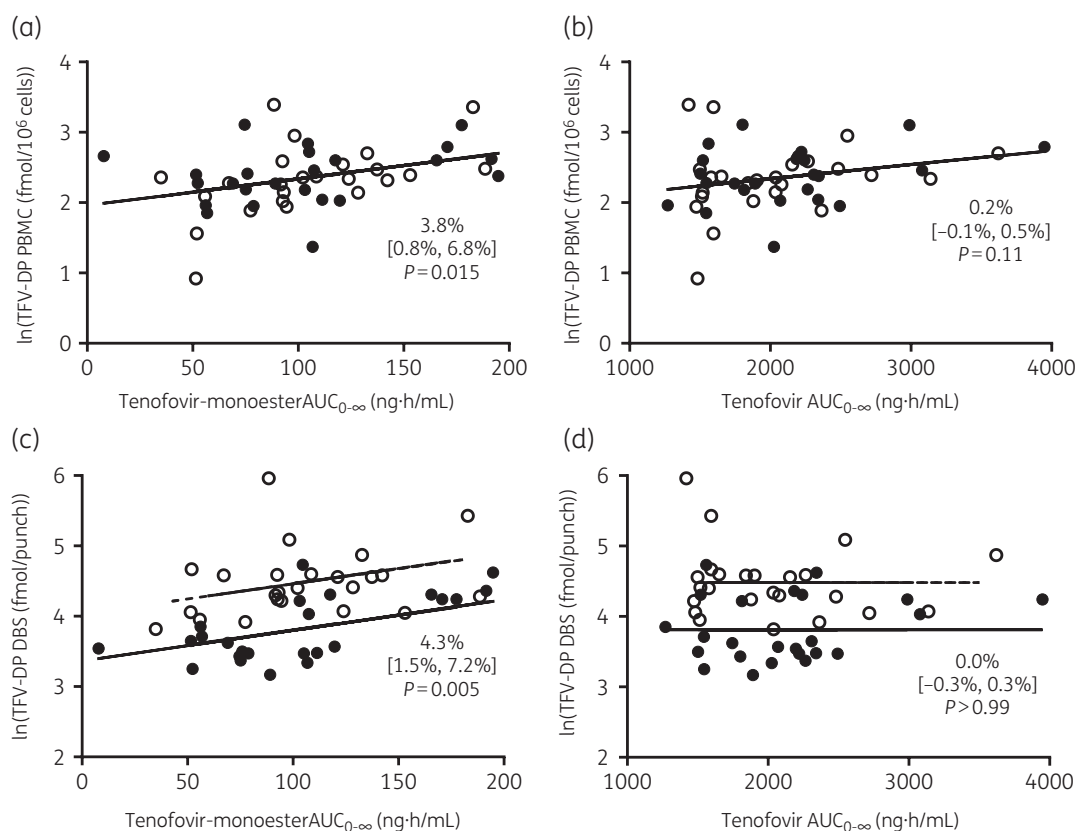
Figure 3. Observed versus predicted plots of tenofovir diphosphate concentrations in PBMC with (a) tenofovir monoester and (b) tenofovir, and tenofovir diphosphate concentrations in DBS with (c) tenofovir monoester and (d) tenofovir. Data are presented as percentage change (95% CI). Filled circles indicate data points from visit 1. Open circles indicate data points from visit 2. Solid lines indicate predictions from all visits in PBMC and visit 1 in DBS, and dashed lines indicate predictions from visit 2.

CES2 is the only form expressed in the intestinal tract, and is also the primary form expressed in kidneys, vaginal epithelia and PBMC in comparison with other identified families.[34,35] Thus, CES enzymes, notably CES2, play a critical role in the initial hydrolysis of

TDF following oral administration and the subsequent delivery of the disoproxil and monoester forms to portal and peripheral blood. Tenofovir disoproxil was not quantified in this study as preliminary work showed it was rapidly degraded when spiked into plasma

(data not shown). It is unlikely that tenofovir disoproxil circulates measurably in the systemic circulation, based on its susceptibility to hydrolysis by esterases and pH changes, in addition to efflux by P-glycoprotein, all limiting systemic tenofovir disoproxil exposures.[2,9,33,36] Tenofovir monoester exposures demonstrated a significant and positive relationship with tenofovir up to 4 h post-dose, but tenofovir monoester only accounted for $\sim$20% of the variability in tenofovir, suggesting that tenofovir monoester may distribute out of plasma and into other compartments before returning into plasma as tenofovir. Additionally, this relationship weakened with extrapolation to infinity as tenofovir monoester was eliminated from systemic circulation after 4 h, whereas tenofovir undergoes prolonged re-distribution and elimination, which influence its profile.

Previous literature on the cell permeability of tenofovir monoester is limited to *in vitro* studies. In Caco-2 cells, tenofovir monoester was able to cross from the apical to the basolateral side, whereas tenofovir transfer was not detected.[37] Within this same experiment, tenofovir disoproxil crossed cell layers at a permeability rate $\sim$10-fold higher than tenofovir monoester, demonstrating the enhanced ability of the disoproxil form to permeate cells. In a separate *in vitro* study, tenofovir monoester accounted for 76% of tenofovir transport across Caco-2 cell monolayers incubated with tenofovir disoproxil.[9] However, the link between systemic circulation of this moiety in humans and its potential influence on cell loading had not been explored, to our knowledge.

In a previous study, we found that tenofovir diphosphate concentrations were increased in individuals receiving TDF-based therapy in combination with ledipasvir/sofosbuvir,[25] which was attributed in part to CES2 inhibition by sofosbuvir leading to higher tenofovir disoproxil and monoester delivery to the portal blood and systemic circulation.[38,39] Consistent with this, a companion paper in this Journal[26] shows elevated tenofovir monoester concentrations concurrent with these increases in a smaller follow-up study. The present analysis examined relationships between tenofovir monoester and intracellular tenofovir diphosphate concentrations in a controlled PK study. A significant and positive relationship was identified between total tenofovir monoester exposure with intracellular tenofovir diphosphate concentrations in PBMC and DBS, consistent with enhanced cell loading leading to higher tenofovir diphosphate *in vivo*. These findings are in contrast to the lack of association between tenofovir exposures and tenofovir diphosphate in DBS and PBMC. Prior research focused on the long persistence of tenofovir in plasma ($t_{1/2}$ $\sim$15 h) and its cellular uptake via endocytosis leading to tenofovir loading of PBMC and subsequent generation of tenofovir diphosphate.[4] However, multiple studies have shown poor cellular penetration of tenofovir.[35,37,40] Further work is needed to define the relative contributions of tenofovir and tenofovir monoester to intracellular tenofovir diphosphate *in vivo*.

These findings have clinical relevance because they provide insight into a previously unrecognized factor influencing intracellular tenofovir diphosphate concentrations, which is the pharmacologically active moiety in PBMC and the moiety used to assess adherence in DBS. TDF has long remained a key component of multiple antiretroviral treatment regimens, and it is currently the only FDA-approved therapy for pre-exposure prophylaxis. In addition, TDF recently became available in generic form and will remain a cornerstone of HIV treatment in resource-limited settings.

A number of demographic factors have been shown to be associated with tenofovir diphosphate concentrations in both healthy volunteers and persons living with HIV, including race, gender, renal function and BMI,[23] and, in those with HIV, the concomitant ART regimen.[18] Our findings suggest that tenofovir monoester exposures should also be considered among the factors that influence tenofovir diphosphate concentrations in PBMC and DBS in individuals receiving TDF. Further research should be pursued to examine biological factors that may influence tenofovir monoester formation, and how this moiety may demonstrate relationships with efficacy or DBS concentrations for adherence in persons on TDF-based therapy.

There are limitations to this study. The primary goal was to establish bioequivalence between the encapsulated versus unencapsulated pills through the measurement of tenofovir and emtricitabine concentrations in plasma, and thus the sample processing and sampling timepoints were selected to accurately capture peak concentrations and the decline of tenofovir and emtricitabine. Given the rapid appearance and decline of the monoester form, additional timepoints may have permitted a more accurate assessment of tenofovir monoester PK in plasma, including the potential to identify a multi-phasic decline similar to that of tenofovir. TDF hydrolysis to the monoester form appears to be slowed under fed versus fasted conditions, potentially due to competition for esterases.[11] All participants were fasted for both PK assessments, and thus variability from this source was not investigated. PBMC and DBS samples were only available at 24 h post-dose in this study, whereas earlier or multiple timepoints may have provided additional kinetic information relative to cell loading. Tenofovir diphosphate concentrations in PBMC and DBS from our study were similar to those published with other single-dose studies.[24,28,41]

In conclusion, this study showed that tenofovir monoester reaches concentrations that approach tenofovir concentrations following oral administration of TDF. This moiety appeared rapidly, but was swiftly eliminated, suggesting quick uptake into cells and metabolism in the liver and blood. Tenofovir monoester was a significant predictor of tenofovir diphosphate concentrations in both PBMC and DBS, suggesting that its PK may be an important source of variability for tenofovir diphosphate within cells. This gives rise to potential clinical relevance for tenofovir monoester, a question that deserves further examination.

Transparency declarations

Disclaimer

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