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RESEARCH PAPER

Pharmacokinetic and Tissue Distribution Profile of Long Acting Tenofovir Alafenamide and Elvitegravir Loaded Nanoparticles in Humanized Mice Model

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ABSTRACT

Purpose Non-adherence to the antiretroviral (ARV) regimen is a critical factor in determining efficacy of ARV drugs for pre-exposure prophylaxis (PrEP). A long-acting parenteral formulation may be an effective alternative to daily oral dosing. A pharmacokinetic and tissue distribution study of drug-loaded nanoparticle (NP) was performed in female humanized $CD34^+$-NSG mice.

Methods Mice received 200 mg/kg each of tenofovir alafenamide (TAF) and elvitegravir (EVG) as free drugs (TAF + EVG solution) or as drug loaded NP (TAF + EVG NP) formulation by subcutaneous (SubQ) administration. Plasma and tissue were collected to determine tenofovir (TFV) and EVG concentrations using LC-MS/MS. Non-compartmental analysis was performed using WinNonlin.

Results SubQ administration of TAF + EVG NP formulation resulted in long residence time and exposure for both drugs. The $AUC_{(0-72h)}$ of TFV and EVG was 14.1 ± 2.0, 7.2 ± 1.8 µg × hr./mL from drugs in solution (free) and the $AUC_{(0-14day)}$ for the same drugs was 23.1 ± 4.4, 39.7 ± 6.7 µg × hr./mL from NPs. The observed elimination half-life ($t_{1/2}$) for TFV of free and NPs were 14.2 h, 5.1 days and for EVG 10.8 h, 3.3 days, respectively.

Conclusion This study documents that a TAF + EVG NP provides sustained release, which can overcome patient non-adherence to dosing and may facilitate prediction of appropriate protective drug concentration for HIV prophylaxis.

ABBREVIATIONS

%EE	Percentage entrapment efficiency
ARV	Antiretroviral
AUC	Area under the curve
COB	Cobicistat
EDTA	Ethylenediaminetetraacetic acid
EVG	Elvitegravir
FRT	Female reproductive tract
FTC	Emtricitabine
HPLC	High performance liquid chromatography
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LLOQ	Lower limit of quantification
NP	Nanoparticle
PBMC	Peripheral blood mononuclear cell
PDI	Polydispersity index
PK	Pharmacokinetic
PLGA	Poly(lactic-co-glycolic acid)
PrEP	Preexposure prophylaxis
PVA	Poly(vinyl alcohol)
RPM	Revolutions per minute
SubQ	Subcutaneous
TAF	Tenofovir alafenamide
TDF	Tenofovir disoproxil fumarate
TFV	Tenofovir

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INTRODUCTION

Combination antiretroviral (ARV) therapy reduces HIV-1 plasma viral load and improves patient life expectancy when used as treatment [1]. But the rate of HIV-1 transmission

remains high, with 2.1 million new infections occurring worldwide in 2015 alone [2]. Based on the efficacy in several pre-exposure prophylaxis (PrEP) trials, US Food and Drug Administration (FDA) has approved daily oral tenofovir disoproxil fumarate (TDF) in combination with emtricitabine (FTC) (Truvada™, Gilead Sciences Inc., USA) for PrEP to prevent HIV-1 infection in men and women [3]. PrEP trials also highlighted the importance of adherence to daily oral regimen to maintain quantifiable tenofovir in plasma, which increased efficacy.

Long-acting parenteral ARV nanoformulations can significantly reduce the dosing frequency [4] and thus improve patient adherence and effectiveness for HIV-1 prevention [5]. Long-term release of antiretroviral drugs is feasible using intravaginal devices (i.e. rings), implants, and injectable formulations [6, 7]. Each of these techniques has drawbacks for the administration of the product. Among these, injectable formulations would offer ease of administration and hence would be advantageous over daily oral dose for prophylaxis. Besides the patient adherence to therapy, a major factor that contributes to protection against HIV-1 is the proper drug concentration in the target mucosal tissues, such as vaginal and rectal tissue [8]. Tissue drug concentrations are affected by many variables such as dose, frequency, mode of administration, tissue permeability, protein binding, and elimination half-life. The effective tenofovir (TFV) plasma concentration to prevent HIV-1 infection has been estimated to be 90–110 ng/mL [9]. However, the concentration at the site of infection (vaginal or rectal) and in the other tissues, has not consistently been reported. Consequently, pharmacokinetic (PK) studies that focus on the mucosal compartments are critical for the future success of PrEP strategies.

Several studies and clinical trials have proved that TDF, an ester prodrug of TFV is effective for prevention of HIV-1 [10–12]. Presently, tenofovir alafenamide (TAF), a FDA-approved nucleotide reverse transcriptase inhibitor prodrug is preferred over TDF due to its comparatively improved side effect profile (less kidney and bone toxicity) for HIV-1 treatment [13]. A long acting formulation containing TAF would be highly desirable. While FTC has proven to be effective for prevention, we hypothesized elvitegravir (EVG), a FDA-approved integrase strand transfer inhibitor, could be a novel drug choice for prevention based on its drug class and clinical pharmacokinetics.

We have previously reported the formulation and characterization of polymeric nanoparticles (NPs) with TAF and EVG entrapped within the polymer [14]. In the present study, we report the pharmacokinetic profile and tissue distribution of TAF and EVG drugs after subcutaneous (SubQ) administration in humanized mice as TAF + EVG NPs and compared with TAF + EVG free drugs (in solution).

MATERIALS AND METHODS

Chemicals

Phosphate buffered saline was purchased from Sigma-Aldrich (St. Louis, MO, USA). TFV reference standard (>99%) was purchased from United States Pharmacopeia, (Rockville, MD). EVG reference standard (>99%) was purchased from Sequoia, (Pangbourne, United Kingdom). Internal standards (TFV-d6 and EVG-d6) were purchased from Toronto Research Chemicals Inc., Canada. Milli-Q water was obtained from in-house Milli-Q water purification system, Millipore, USA. LC-MS grade methanol, acetonitrile, formic acid and trifluoroacetic acid were purchased from Fisher Scientific, USA.

ARV Loaded NP Preparation and Characterization

NP preparation and characterization was carried according to our previously reported publication [14]. Briefly, to formulate TAF + EVG NPs, we followed oil-in-water (o-w) emulsion methodology. The organic phase (dichloromethane), containing PLGA and PF-127 at 1:1 ratio along with TAF and EVG at 50 mg/100 mg PLGA, was emulsified with aqueous phase of 1% PVA solution, followed by sonication. The organic phase was evaporated and the emulsion was clarified using a dialysis membrane (Slide-A-lyzer, 10 K, Pierce, Inc.) and then freeze-dried. The physicochemical characteristics (size, poly dispersity index-PDI and surface charge) of the TAF + EVG NPs were analyzed using a Zeta-sizer Instruments and the percentage drug entrapment efficiency (%EE) was analyzed by HPLC following slight modification of previously published procedure [15]. (Mobile phase: 25 mM potassium dihydrogen phosphate: acetonitrile (45:55 v/v); TAF and EVG: absorbance maximum was at 260 and 313 nm respectively; retention time 4 and 20 min respectively). Intra-day and inter-day variability was <15%.

To evaluate stability of TAF + EVG NPs over time (1 year), 5 mg of freeze dried TAF + EVG NPs were dispersed in 1 mL 40% DMSO solvent at time 0 (freshly prepared and freeze dried) and at time 1 year (same batches that were used in this study, 1 year old freeze dried NPs stored at 4°C). The size, PDI and % EE were evaluated as stated above. Mean ± standard error (SE) of mean obtained from four different batches of TAF + EVG NPs was calculated and statistical significance test was performed to evaluate stability.

Pharmacokinetic study design

Female humanized CD34$^+$ NOD.Cg-Prkdc^{scid}IL2rg^{tm1Wjl}/Szj (NOD/SCID/IL2rgnull, CD34$^+$-NSG) mice were purchased from Jackson Laboratory (Bar Harbor, MA) and allowed to acclimate to the animal facility for seven days. Mice were

divided into two groups, one group of 15 mice (3 mice/time point) were injected SubQ with 200 mg/kg (each TAF and EVG) as TAF + EVG NP in 1 mL of 5% dextrose. Another group of 15 mice (3 mice/time point) were injected SubQ with 200 mg/kg (each TAF and EVG) of free drug in 1 mL of 5% dextrose (TAF + EVG solution). Two SubQ injections of 0.5 mL were given with a gap of 4 h, to avoid dose volume stress on mice. At 1, 4, 7, 10 and 14 days after injection of TAF + EVG NP and at 4, 8, 24, 48 and 72 h after injection of free TAF + EVG in solution, mice were sacrificed by carbon dioxide inhalation and cervical dislocation. Institutional Animal Care and Use Committee approved protocol (#0989) and procedures were followed.

Blood and Tissue Collection

Whole blood was collected in EDTA containing blood collection tubes, centrifuged at 2000 RPM for 10 min and plasma harvested. The tissues included liver, kidney, spleen, lymph node, female reproductive tract (FRT, consisting ovaries and fallopian tube), vagina, colon, and injection site were harvested, placed into tubes and frozen at $-80°C$ until analysis by LC-MS/MS.

Determination of Drug Concentration in Plasma and Tissue

Plasma and tissues samples were analyzed for drug concentration by following our previously reported method [16]. Briefly, 100 µL aliquot of plasma or tissue homogenate (tissues were homogenized in deionized water [1:1 wt/vol] using beads) was mixed with 25 µL of internal standard spiking solution followed by 100 µL of 1% trifluoroacetic acid and samples were vortexed for 30 s. The discovery C_{18} solid phase extraction cartridges were equilibrated with 1 mL of methanol followed by water and samples were loaded. Cartridges were washed with water followed by 5% methanol and eluted with 1 mL of methanol. Eluent was evaporated to dryness under the stream of nitrogen at 40°C, reconstituted with 100 µL of 50% acetonitrile in water and 5 µL was injected into the LC-MS/MS instrument. The lower limit of quantification (LLOQ) for TFV and EVG was 10 and 5 ng/mL respectively in both plasma and tissue. If the observed concentrations were < these LLOQ values, the results were reported as half the LLOQ.

An Agilent 1200 HPLC system (Agilent Technologies, CA, USA) coupled with AB Sciex API 3200 Q Trap with an electrospray ionization source (Applied Biosystems, Foster City, CA, USA) was used. Chromatographic separation was carried out on Restek Pinnacle DB Biph (2.1 mm × 50 mm, 5 µm) column with isocratic mobile phase consisting of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) (48:52 v/v) at a flow rate of 0.250 mL/min. The dynamic

range of the validated assay was 10 to 4000 ng/mL for TFV and 5 to 2000 ng/mL for EVG.

Determination of Pharmacokinetic Parameters of Drug Loaded NPs and Free Drug

The measured concentrations of TFV and EVG were subjected to noncompartmental pharmacokinetic analysis using Phoenix WinNonlin software version 6.4 (Certera Inc., Princeton, N.J.). The following pharmacokinetic parameters were calculated from the plasma and tissue concentration-time data: maximum drug concentration (C_{max}), time at which C_{max} was observed (t_{max}), area under the concentration time data from 0 to last concentration measured ($AUC_{(0-t)}$), half-life of elimination ($t_{1/2}$), volume of distribution (V/F) and total body clearance (CL/F).

RESULTS

Characterization of NPs

The NPs physicochemical characterization has been reported in our earlier article [14]. Briefly, the o-w emulsion methodology resulted in uniform well-defined TAF + EVG NPs having 221.6 ± 6.1 nm size, with PDI; a measure of the NP size variability of 0.107 ± 0.01 (less than <0.2, i.e. uniform size-distribution), and surface charge of -19.2 ± 1.7 ($n = 5$). The %EE of TAF and EVG into the NPs were $57.28 \pm 4.0\%$ and $49.5 \pm 4.3\%$, respectively ($n = 4$). The statistical significance data shows, freeze-dried TAF + EVG NPs are stable over 1 year at 4°C (Table I).

Pharmacokinetic Profile and Tissue Distribution Profile of ARV NPs and Free Drug

The sustainability and extent of permeability of ARV drug loaded NPs into tissues was evaluated by comparing the plasma and tissue concentrations of free ARV drugs with ARV drugs in the nanoformulation over time. The mean plasma and tissue concentration versus time profiles of free and

Table I Mean ± Standard Error (SE) Stability Data of TAF + EVG NPs over Time

Parameter	At time '0'	At time '1 year'	Statistical significance
Size (nm)	221.6 ± 6.09	210.05 ± 10.27	ns
PDI	0.107 ± 0.012	0.148 ± 0.007	ns
% EE-TAF	57.28 ± 3.96	55.15 ± 5.27	ns
% EE-EVG	49.46 ± 4.26	46.33 ± 4.87	ns

ns: Statistically not significant

nanoformulation after SubQ injection are shown in Figs. 1 and 2. The mean plasma pharmacokinetic parameters of free and nanoformulation are presented in Table II. The calculated mean plasma C_{max} for TFV (263.6 ng/mL) obtained from the nanoformulation was >110 ng/mL, which has been theorized to be the effective plasma TFV concentration to prevent HIV-1 infection [9]. The mean plasma C_{max} of EVG (577.7 ng/mL) was greater than that achieved with free drug (350.4 ng/mL). The $t_{1/2}$ of the nanoformulation was 5.1 and 3.3 days for TFV and EVG, which is approximately 8- and 5-fold higher compared to free TAF and EVG respectively (Table II), suggesting long residence time of both drugs in biological fluids. The lower clearance (CL/F) and higher $AUC_{(0-t)}$ for ARV in nanoformulation indicates the NP formulation prolongs ARV residence time compared to the same dose of free drugs.

The mean pharmacokinetic parameters of TFV and EVG for all tissues are presented in Table III. The tissue concentrations especially at the anatomic sites of infection (vagina and colon) demonstrated a detectable TFV and EVG concentrations even on day 14 when administrated as nanoformulation. Overall, in majority of the tissues studied, nano-encapsulated ARV demonstrated drug concentrations on day 10 equal to or higher than the 72 h concentration of the drugs when administered as free. In all tissues, the $AUC_{(0-t)}$ of ARV in nanoformulation was always higher than the free drugs. From the C_{max},

$AUC_{(0-t)}$ and $t_{1/2}$ of the tissues, it is evident that the drug exposure with nanoformulation is efficient and prolonged. Also, the tissue drug concentration vs time profile for both TFV and EVG at the SubQ injection site, indicates that nanoformulation prolongs the diffusion from the administration site. Some of the tissue concentrations from nanoformulation were found to be statistically significant compared to drugs in solution. The less significance can be correlated to staggered approach and scarce sample size.

No adverse events or mortality was observed and mice were healthy after injecting the formulation through completion of PK study. While collecting mice injection site samples for analysis of drug concentration on different days [1, 4, 7, 10, 14] after sacrificing mice suggests that a patch was formed and lasted till day 10, which was disappeared in Day 14 mice.

DISCUSSION

The primary objective of this study was to evaluate and compare pharmacokinetic profiles and biodistribution of drug loaded NPs versus free drug with emphasis on HIV-1 prevention. All the HIV-1 prevention trials with TDF/FTC demonstrated greater efficacy with measurable TFV concentration in plasma [10–12, 17]. For PrEP, plasma TFV and PBMC active metabolite (TFV-diphosphate) concentrations are acceptable scale for
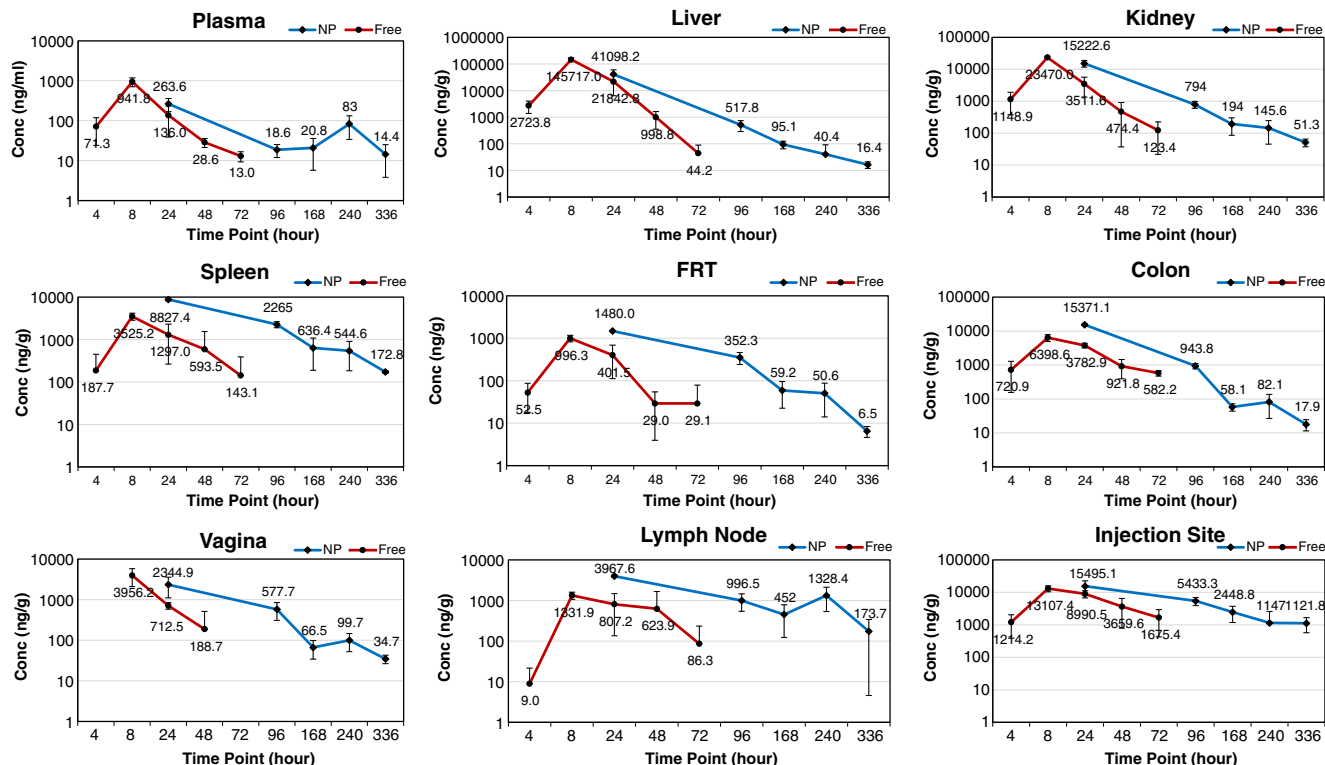
Fig. 1 Plasma and tissue concentrations of TFV after subcutaneous administration of 200 mg/kg dose of free and NP formulations to mice. Error bars represent standard deviation of three mice measurements by LC-MS/MS. In graph legend, "Conc" represent concentration, "NP" represents TAF + EVG NP and "Free" represents TAF + EVG in solution.
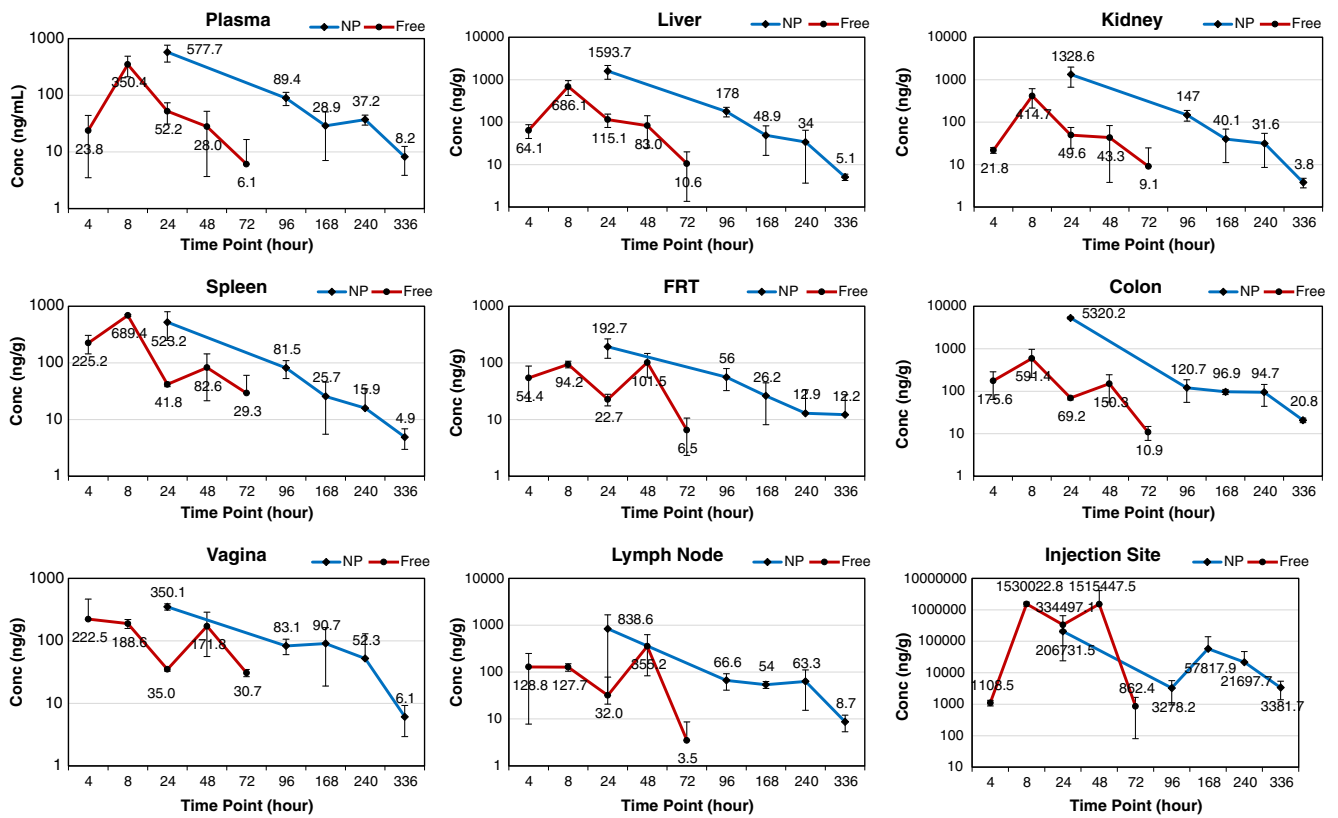
Fig. 2 Plasma and tissue concentrations of EVG after subcutaneous administration of 200 mg/kg dose of free and NP formulations to mice. Error bars represent standard deviation of three mice measurements by LC-MS/MS. In graph legend, "Conc" represent concentration, "NP" represents TAF + EVG NP and "Free" represents TAF + EVG in solution.

estimating protective drug levels [18]. The Partners-in-PrEP clinical trial reported plasma TFV levels >40 ng/mL was significantly protective [19]. The accurate protective concentration at the site of infection necessary for full protection against HIV-1 has not been established. Therefore, protective drug concentration at site of infection must be defined. The present study showed that the NP formulation achieved the reported protective plasma concentration (~100 ng/mL). The tissue exposure was efficient in all the tissues studied with NP formulation compared to free drugs in solution (TFV $AUC_{(0-t)}$ was 62.6 ± 12.4, 169.0 ± 43.6, EVG $AUC_{(0-t)}$ was 8.8 ± 1.9, $34.0 \pm 4.7 \mu g \times hr./g$ for free drugs and NP formulation respectively at vagina). A comparison of vaginal AUC values of both drugs from the

present study indicate prolonged exposure of ARV drugs. Indeed, the vaginal AUC from the NP formulation was approximately 2.7–3.9 times higher for both TFV and EVG compared to the free drugs in solution administered to the mice. The better tissue exposure is important for the PrEP agents where the initial virologic events are localized [20].

Previous studies on pharmacokinetic parameters of TFV have been investigated in humanized mice. Female mice received 61.5 mg/kg TDF orally had a peak TFV level of 1990 ng/mL, AUC of 11,251 ng × h/mL and $t_{1/2}$ of 17 h [8]. In our study TAF + EVG NP formulation demonstrated plasma TFV AUC of 23,134 ng × h/mL and $t_{1/2}$ of 5.1 day for mice receiving 200 mg/kg SubQ (Table II). The overall drug

Table II Mean ± Standard Error (SE) Plasma Pharmacokinetic Parameters of Free and Nanoformulated TAF and EVG	Parameter	Units	Tenofovir		Elvitegravir	
			Free	NP	Free	NP
	C_{max}	ng/mL	941.8 ± 137.1	263.6 ± 67.6	350.4 ± 79.9	577.7 ± 136.0
	t_{max}	Hour (h)/Day	8 h	1 Day	8 h	1 Day
	$AUC_{(0-t)}$	$\mu g \times hr./mL$	14.1 ± 2.0	23.1 ± 4.4	7.2 ± 1.8	39.7 ± 6.7
	$t_{1/2}$	Hour (h)/Day	14.2 h	5.1 Day	10.8 h	3.3 Day
	V/F	L/kg	285.3	1369.9	427.7	553.4
	CL/F	L/h/kg	14.0	7.8	27.6	4.9

Table III Mean ± Standard Error (SE) Tissue Pharmacokinetic Parameters of Free and Nanoformulated TAF and EVG

Tissue	Formulation	C_{max} (ng/g)	AUC$_{(0-t)}$ (μg × hr./g)	$t_{1/2}$
Spleen	TFV-Free	3525.2 ± 389.0	83.8 ± 19.1	15.1 h
	TFV-NP	8827.4 ± 504.7*	686.6 ± 35.0*	2.9 Day
	EVG-Free	689.4 ± 14.3	11.6 ± 1.1	16.8 h
	EVG-NP	523.2 ± 194.5	34.4 ± 9.5	2.6 Day
Lymph Node	TFV-Free	1331.9 ± 154.7	48.4 ± 16.7	14.9 h
	TFV-NP	3967.6 ± 214.1*	414.7 ± 64.2	3.7 Day
	EVG-Free	355.2 ± 157.0	11.0 ± 3.8	9.3 h
	EVG-NP	838.6 ± 472.2	54.7 ± 23.1	2.4 Day
Colon	TFV-Free	6398.6 ± 850.1	175.9 ± 13.9	17.8 h
	TFV-NP	15,371.1 ± 782.7*	817.7 ± 38.5*	1.4 Day
	EVG-Free	591.4 ± 219.8	13.3 ± 3.0	12.5 h
	EVG-NP	5320.2 ± 207.8*	280.0 ± 11.2*	4.2 Day
Vagina	TFV-Free	3956.2 ± 1066.2	62.6 ± 12.4	9.3 h
	TFV-NP	2344.9 ± 874.0	169.0 ± 43.6	2.2 Day
	EVG-Free	222.5 ± 141.8	8.8 ± 1.9	30.8 h
	EVG-NP	350.1 ± 28.0	34.0 ± 4.7	1.8 Day
FRT	TFV-Free	996.3 ± 100.6	27.5 ± 7.4	12.7 h
	TFV-NP	1480.0 ± 2.6	105.2 ± 5.3*	1.9 Day
	EVG-Free	101.5 ± 26.4	4.6 ± 0.8	20.1 h
	EVG-NP	192.7 ± 41.7	16.8 ± 2.5	4.5 Day
Liver	TFV-Free	145,717.0 ± 13,183.4	2215.3 ± 362.1	5.4 h
	TFV-NP	41,098.2 ± 13,098.0	2021.0 ± 628.8	2.8 Day
	EVG-Free	686.1 ± 151.0	15.3 ± 3.6	10.3 h
	EVG-NP	1593.7 ± 403.3	95.9 ± 19.5	2.1 Day
Kidney	TFV-Free	23,470.0 ± 1201.1	383.2 ± 67.1	9.9 h
	TFV-NP	15,222.6 ± 2436.0	816.5 ± 117.3	2.7 Day
	EVG-Free	414.7 ± 114.7	8.5 ± 2.2	12.3 h
	EVG-NP	1328.6 ± 467.3	80.1 ± 22.5	2.0 Day
SubQ site	TFV-Free	13,107.4 ± 1558.2	445.1 ± 55.2	19.8 h
	TFV-NP	15,495.1 ± 5025.0	1461.5 ± 255.4	4.3 Day
	EVG-Free	1,530,022.8 ± 129,742.5	60,772.1 ± 36,075.5	7.0 h
	EVG-NP	206,731.5 ± 15,157.8*	25,726.9 ± 9858.3	1.2 Day

*indicates $p < 0.05$. FRT: female reproductive tract; SubQ site: injection site of ARV administration

exposure demonstrated by the tissue: plasma AUC ratios (T:P ratio) for TFV were 7.3 and 35.3 for vaginal and colon tissues respectively. This observed differential accumulation in various tissues may be due to distinct cellular uptake of nanoparticles. Rhesus macaques administered 50 mg/kg TAF/FTC/EVG/ COB orally demonstrated peak TFV levels of 1326 ng/mL and AUC of 9934 ng × h/mL [21]. The plasma AUC for EVG in macaques (Rhesus, 50 mg/kg) was reported as 4012 ng × h/mL [22]. In contrast, the plasma TFV AUC and EVG AUC in the CD34$^+$-NSG mice in this study were approximately 2.3 and 9.9-fold higher than AUC observed in macaques with equivalent dosing.

The use of single drug may not be adequate to confer full protection, hence combination of ARV drugs with different mechanism of action were selected. We have previously published the addition of EVG *in vitro* is synergistic to TFV for HIV-1 protection [14]. However, further trials are appropriate in larger animal models for the ARV NPs to prove a link between tissue drug concentration and efficacy.

Although the goal of this project was to evaluate and compare the pharmacokinetic and tissue distribution profile of free and nanoformulated ARV drugs for HIV-1 prevention, it is conceivable that this formulation could be used in both post-exposure prophylaxis as a one-time administration, as well as a treatment regimen. The back-up systemic drug and biodistribution of drug to sanctuary sites (lymph node, spleen and brain etc.) is an effective strategy to prevent and eradicate virus within the biological system [23, 24], which was accomplished in this study. Sanctuary sites include areas within the body with reduced ARV penetration compared to plasma

[23]. Future studies will be aimed at analyzing metabolite (TFV-diphosphate) concentration in PBMC and tissue CD4 cells to study cellular uptake and pharmacokinetics of ARV drugs via the nanoformulation.

In conclusion, most of the prevention clinical trials conducted has claimed non-adherence is a critical variable for protection from HIV-1 infection [25]. These experiments provide evidence for sustained release of drugs from our nanoformulation, which could potentially reduce dosing frequency for prevention of HIV-1 infection. However further studies are necessary in larger animal model with multiple dosing to confirm these results.

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